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Comparative analyses of the function of alpha- and beta-catenin in cerebral cortical development

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Abstract

Precursor proliferation and differentiation act in coordination with morphogenic processes to build the cerebral cortex during development. The control of morphogenesis and proliferation has therefore to be tightly linked. One example of such a link is the simultaneous function of β-catenin in canonical Wnt signalling and adherens junction (AJ) formation. In this work, I investigated the role of β-catenin during the development of the cerebral cortex and compared it to that of α-catenin to reveal which of the developmental functions of β-catenin can be assigned to its participation in AJ formation. The analyses performed show that β-catenin influences the size of the cortical precursor pool by controlling cell cycle exit and cell death but its role in these processes decreases with development. In the absence of β-catenin, some cortical precursor cells still generate intermediate progenitors and neurons albeit in small numbers, suggesting that not all cortical precursor cells rely on canonical Wnt signalling to remain proliferating. Neuronal birth dating analyses however revealed that slight alterations occurred during neuronal subtype specification in the β-catenin deficient cortex. In addition to the aberrations in proliferation and differentiation, β-catenin deficient radial glial cells were highly impaired in their morphology; they no longer showed their characteristic bipolar shape. Similar defects were observed in the α-catenin deficient cortex, arguing that this phenotype resulted from abnormalities in the AJs, as both molecules, α- and β-catenin, contribute to the formation of this structure. Surprisingly however, electron microscopy demonstrated that the mutant cells maintained AJs. Thus the morphological alterations observed in the mutant cortices could not have been caused by a lack of AJs but may have resulted from the impaired connection of the AJs with the cytoskeleton, weakening the overall cohesiveness of the cortical tissue. The loss of radial glial morphology resulted in the disturbed cortical architecture that was observed in the absence of β-catenin and also α-catenin, with neurons of different subtypes distributed throughout the cortex in contrast to the precisely laminated architecture of the wild type cortex. In contrast to the lack of β-catenin, loss of α-catenin led to an increase in precursor cells that turned out to be due to a transiently accelerated cell cycle, promoted by the increased expression of CyclinD1. Micro Array analyses revealed that this was not caused by ectopic activation of an entire signalling pathway, although increased TCF/LEF transcriptional activity was observed at the most rostral levels of the cortex, where the proliferation phenotype was most prominent. Despite this, intermediate progenitors were generated and neuronal subtypes correctly specified in the α-catenin deficient cortex. Thus,

the loss of α-catenin interferes with cell cycle length and radial glial morphology but both do not impair precursor fate or neurogenesis.

This work presents evidence that β-catenin participates in two different aspects of cortical development, the expansion and the lamination of the cerebral cortex. The former aspect it fulfils by transducing canonical Wnt signalling whereas the latter it serves by participating in the connection of the AJs to the cytoskeleton, as evident from the comparative analyses between β- and α-catenin deficient mutants.

Zusammenfassung in deutscher Sprache

Die Entwicklung des Gehirns ist gekennzeichnet durch die Abfolge von Zellteilung, Zelldifferenzierung und Zellreifung. Diese einzelnen Stadien sind zudem mit morphologischen Veränderungen der Gehirnzellen verbunden, so dass die Entwicklung des Gehirns wie die anderer Organe auch auf der ineinander greifenden Kontrolle von Identität und Gestalt der Zellen beruht. Während der Embryogenese entwickelt sich das Zentralnervensystem durch massive Zellteilung aus dem, eine Zellschicht umfassenden Neuroepithel. Nicht zuletzt durch die morphologische Veränderung individuellen Zellen sowie des Zellverbands als Gesamtheit entsteht im Endhirn die 6 neuronale Schichten umfassende Großhirnrinde. Im Gegensatz zur Kontrolle morphologischer Vorgänge, die überwiegend auf molekularer Ebene statt findet, werden Zellteilung und Differenzierung prävalent durch Gen-transkription gesteuert. Beide Prozesse sind jedoch eng verbunden, unter anderem durch Proteine die sowohl an der Gen-transkription als auch an der Zellgestaltung beteiligt sind. β-catenin gehört zu diesen Proteinen, es vermittelt die durch extrazelluläre WNT Proteine induzierte Gen-transkription, nimmt aber auch strukturelle Aufgaben in der Zelladhäsion wahr. Zusammen mit α-catenin stellt β-catenin die Verbindung zwischen dem Adhäsionsgürtel und dem Zytoskelett her. α-catenin ist ein dem β-catenin verwandtes Protein teilt aber nicht dessen transkriptionelle Eigenschaften. Ziel dieser Arbeit war es, in einem vergleichenden Ansatz, die Funktion von β-catenin und α-catenin während der Embryonalentwicklung der Großhirnrinde zu untersuchen. Zu diesem Zweck wurden drei verschiedene β-catenin Mausmutanten untereinander verglichen, in welchen β-catenin zu unterschiedlichen Zeitpunkten der cerebralen Entwicklung oder in unterschiedlichen Regionen der Großhirnrinde eliminiert wurde. Darüber hinaus sollte untersucht werden welche der Funktionen in der Entwicklung der Großhirnrinde den transkriptionellen und welche den strukturellen Eigenschaften β-catenins zugeordnet werden können. Daher wurden die Untersuchungen der β-catenin Mausmutanten durch den Vergleich mit einer α-catenin Mausmutante erweitert.

Die Untersuchung der drei unterschiedlichen β-catenin Mutanten zeigte, dass β-catenin die Anzahl der Vorläuferzellen bestimmt, indem es kontrolliert ob eine Vorläuferzelle den Zellzyklus verlässt und in eine Nervenzelle differenziert oder aber durch kontinuierliche Zellteilung weitere Vorläuferzellen bildet. Darüber hinaus wirkt β-catenin dem apoptotischen Zelltod entgegen. Es zeigte sich, dass die Bedeutung β-catenins für die Kontrolle der

Zellteilung und -differenzierung mit fortschreitender Entwicklung abnimmt, dass aber auch schon zu frühen embryonalen Stadien nicht alle Vorläuferzellen auf den Entzug von β-catenin durch vorzeitige Differenzierung reagieren. Das lässt darauf schließen, dass sich cerebrale Vorläuferzellen in ihrer Sensibilität für WNT induzierte Proliferation unterscheiden und dass diese mit fortschreitender Entwicklung abnimmt. Des Weiteren ergab die Analyse neuronaler Subklassen und ihrer zeitlichen Entstehung, dass der Verlust von β-catenin oder die durch ihn ausgelösten Veränderungen der Vorläuferzellen, die neuronale Spezifikation beeinträchtigen und den 6-schichtige Aufbau der Großhirnrinde unterbinden.

Vorläuferzellen ändern jedoch nicht nur ihr Teilungsverhalten durch die Abwesenheit von β-catenin sondern zeigen eine starke Veränderung ihrer Morphologie. Neurale Vorläuferzellen sind durch eine polare, längliche Struktur gekennzeichnet, da sie lange radiale Fortsätze bilden, mit denen sie die Großhirnrinde von der ventrikulären bis zur äußeren Oberfläche durchmessen. Die Fortsätze, die sich zum Ventrikel hin erstrecken, sind durch Adhäsionsgürtel verbunden, an deren Bildung β-catenin beteiligt ist. Zellen, die kein β-catenin produzieren, bilden lediglich kurze Fortsätze und sind generell von rundlicher Gestalt. Eine ähnliche Veränderung der Zellmorphologie zeigten neuronale Vorläuferzellen der α-catenin Mutante, die ebenfalls keine 6-schichtige Struktur der Großhirnrinde ausbildete. Da beide Proteine an der Bildung des Adhäsionsgürtels und seiner Verbindung zum Zytoskelett beteiligt sind, lassen die ähnlichen morphologischen Veränderungen darauf schließen, dass eine beeinträchtigte Adhäsion Grund für die veränderte zelluläre und auch cerebrale Morphologie ist. Interessanterweise bleiben Zellkontakte jedoch erhalten wenn α- oder β-catenin fehlen, daher ist anzunehmen dass der Verlust von α- oder β-catenin Zelladhäsion als solche zwar zulässt, die Verbindung zum Zytoskelett jedoch derart schwächt, dass die bipolare Morphologie der Zellen nicht aufrecht erhalten werden kann und damit die geordnete Anordnung der Zellen verloren geht. Da Neurone die Fortsätze neuraler Vorläuferzellen als Fortbewegungsstütze verwenden, liegt es nahe, dass der Verlust der radialen Fortsätze die Urasche der unzulänglichen Strukturierung der Großhirnrinde der α-cantenin so wie der β-catenin Mutanten darstellt.

Im Gegensatz zu β-catenin Mutanten, die aufgrund der verfrühten Differenzierung neuraler Vorläuferzellen eine kleinere Großhirnrinde haben, ist die Großhirnrinde der α-catenin Mutanten in ihrem Umfang stark vergrößert. Detaillierte Untersuchungen der Zellteilung und Differenzierung sowie des apoptotischen Zelltods machten deutlich, dass die Vergrößerung der Großhirnrinde auf eine vorübergehende Beschleunigung des Zellzyklus zurückzuführen ist, welche kurz nach der Deletion von α-catenin auftritt. Eine Analyse der gesamten

Genexpression neuronaler Vorläuferzellen, die nicht mehr über α-catenin verfügen, zeigte, dass diese Zellen eine erhöhte Expression von CyclinD1 aufwiesen, ein Protein, welches für seine teilungsfördernde Aktivität bekannt ist. Diese erhöhte Expression war jedoch, wie auch die Beschleunigung des Zellzyklus, vorübergehend, da sie nur unmittelbar nach dem Verlust von α-catenin festgestellt werden konnte. Die dennoch erhebliche Vergrößerung der Großhirnrinde veranschaulicht die Konsequenzen einer vorrübergehenden Beschleunigung des Zellzyklus, welche zu einer andauernden Erhöhung der Gesamtzahl der Vorläuferzellen führt. Diese bilden wiederum die vergrößerte Struktur der Großhirnrinde aus. Obwohl die Analyse der Genexpression der α-catenin Mutante keine Veränderung eines spezifischen Signal-Transduktions-Wegs aufzeigte, wurden in rostralen Bereichen der sich entwickelnden Großhirnrinde eine erhöhte TCF/LEF basierte Gentranskription festgestellt. Diese stellt eine der bekannten Steuerelemente der Expression von CyclinD1 dar. Darüber hinaus war die, durch den Verlust von α-catenin bedingte, Expansion der Großhirnrinde rostral besonders ausgeprägt. Diese Ergebnisse lassen vermuten, dass die erhöhte TCF/LEF basierte Gentranskription zwar nicht die alleinige Ursache der Zellzyklusbeschleunigung darstellt, aber vor allem rostral maßgeblich zu dieser beiträgt.

Trotz der vergrößerten Großhirnrinde war die neuronale Spezifizierung in diesen Mutanten nicht beeinträchtigt, so dass neuronale Subtypen aller cerebralen Schichten gefunden wurden, wenn auch nicht in ihrer üblichen Anordnung. Die Zellmorphologie der Vorläuferzellen ist daher von entscheidender Bedeutung für die Anordnung der Nervenzellen in der Großhirnrinde, nicht aber für deren Bildung und Spezifizierung.

Die in der vorliegenden Arbeit beschriebenen Experimente legen dar, dass β-catenin, als zellulärer Vermittler des WNT Signal-Transduktions-Wegs die Anzahl cerebraler Vorläuferzellen und damit die Größe der Großhirnrinde mitbestimmt. Darüber hinaus konnte anhand von vergleichenden Untersuchungen gezeigt werden, dass β-catenin sowie auch α-catenin essentiell zur Aufrechterhaltung der cerebralen Struktur sind, auch wenn ihr Verlust die Zelladhäsion per se nicht unterbindet. Der Verlust von α-catenin jedoch wirkte sich beschleunigend auf die Zellteilung aus, so dass die Großhirnrinde der α-catenin Mutante die bis zu 3 fache Masse einer regulären erreichte. Weder die erhöhte Zellteilung noch die mangelnde Strukturierung beeinträchtigten jedoch die neuronale Spezifizierung. Demzufolge koordiniert β-catenin gleichzeitig zwei unterschiedliche Dimensionen der Groshirnrinde, ihre Größe, da die Anzahl der Vorläuferzellen von der Aktivität des WNT Signal-Transduktions-Wegs abhängt, und ihren zellulären Aufbau, der auf der radialen Morphologie der Vorläuferzellen basiert.

Introduction

The development of the mammalian brain relies on balanced morphogenetic processes in which proliferation and differentiation are tightly coordinated. By the control of precursor proliferation and differentiation, the expansion of the brain and its neuronal as well as glial diversity are determined. Morphogenetic processes in turn create the shape of the brain and its complex interconnectivity.

In the present thesis, I studied the function of two catenin molecules, β-catenin and α-catenin in these biological processes during the development of the cerebral cortex. I used a comparative approach to distinguish the morphogenetic functions shared by both molecules from their distinct roles in proliferation and differentiation.

Generation of the cerebral cortex from the neuroectoderm

Three different germ layers are formed during early embryogenesis, the endo-, meso- and ectoderm, of which the latter gives rise to the nervous system and the epidermis. The prospective epidermis expresses bone morphogenetic proteins (BMP), determining its own epidermal fate, whereas the Spemann's organizer, secreting BMP inhibitors, induces the formation of the neuroectoderm. After gastrulation, the tissue of the Spemann's organizer contributes to the notochord that continuously participates in the patterning of the central nervous system (CNS).

The entire nervous system originates from the neuroectoderm but separates into the central and the peripheral nervous system (PNS) upon neurulation. During this process the neural plate, localized dorsal to the notochord, wedges towards ventral and elevates its edges to fuse at the dorsal midline. By these morphological changes the neural plate transforms into the neural tube while the cells of the neural folds delaminate from the neuroectoderm and form the neural crest cells. The neural plate gives rise to the CNS whereas the neural crest cells generate the PNS and parts of the craniofacial skeleton. This first phase of neurulation is followed by a comprehensive rearrangement of cells and the extension of the neural tube along the dorso-ventral (DV) and the anterior-posterior (AP) axis. Subsequently, the neural tube develops along its AP axis into forebrain, midbrain, hindbrain and spinal cord. These regions become progressively specified through the establishment of local organizers and cellular borders, allowing a partially autonomic development of individual regions of the

CNS. The midbrain and hindbrain, for example, are separated by the mid-hindbrain-boundary (MHB) that functions as a signalling centre but also as an impassable border.

The forebrain develops at the most anterior pole of the neural tube and can be subdivided into the telencephalon (anterior) and the diencephalon (posterior). The telencephalon is further subdivided along the DV axis into the pallium/cerebral cortex and the subpallium/ganglionic eminences with the pallial-subpallial-boundary sharply separating both. The dorsal telencephalon contains the anlagen of the neocortex, the hippocampus, the olfactory cortex and parts of the amygdala, whereas the ventral telencephalon gives rise to the striatum, the pallidum and different parts of the amygdala. During early development the identity of the cortex and the ganglionic eminences becomes defined by specific gene expression patterns. Transcription factors (TF) determining dorsal fate, such as the Pax6 or the empty spiracles homologs (Emx) 1 and 2, are expressed in cortical progenitor cells (Walther and Gruss 1991; Simeone et al. 1992; Stoykova and Gruss 1994; Mallamaci et al. 1998; Bishop et al. 2000) and those specifying ventral fate, such as Nkx2.1, Gsh2 and the mammalian achaete-scute complex homolog (Mash) 1 are expressed in progenitor cells of the ganglionic eminences (Guillemot and Joyner 1993; Corbin et al. 2000; Toresson et al. 2000, for review see: Jessell and Sanes 2000; Takahashi and Liu 2006) In the spinal cord, SHH secreted from the notochord and the floorplate and BMPs secreted from the roofplate oppose each other to establish progenitor populations along the DV axis that differ in their TF expression and their neuronal fate (Tanabe and Jessell 1996; Briscoe et al. 2000) In the developing telencephalon in contrast, the interactions between extrinsic signalling and TFs appear to be more complex. Similar to the spinal cord, SHH induces ventral progenitor fate in the telencephalon, positively regulating the expression of Nkx2.1 and other ventral TFs that are essential to prevent dorsalisation and mis-specification of ventral progenitor cells (Shimamura and Rubenstein 1997; Kohtz et al. 1998; Corbin et al. 2000; Toresson et al. 2000, for review see: Lupo et al. 2006) The ventralising function of SHH is inhibited by Gli3, expressed in the dorsal telencephalon that positively regulates the expression of Emx genes (Theil et al. 1999; Rallu et al. 2002; Kuschel et al. 2003). In addition, Emx2 has been found to be regulated by Wnt signalling as well as by BMPs (Theil et al. 2002; Muzio et al. 2005). These regulations however are bi-directional as Emx2 has been reported to promote Wnt signalling (Muzio et al. 2005) and also BMP signalling by repressing the BMP antagonist Noggin (Shimogori et al. 2004).

Wnt ligands and BMPs are highly expressed in the cortical hem that was therefore suggested to serve as a signalling centre of the dorsal telencephalon (Grove et al. 1998) together with the

anterior neural ridge and the anti-hem, localized at the pallial-subpallial boundary (for review see: Takahashi and Liu 2006). Wnt signalling from the cortical hem is essential for the development and the specification of the hippocampus and the dentate gyrus (Galceran et al. 2000), whereas BMP signalling controls dorsal midline patterning and the specification of the Choroid plexus (Panchision et al. 2001; Hebert et al. 2002). The antihem expresses fibroblast growth factor (FGF) as well as epidermal growth factor (EGF) proteins and neuregulin. It is lost in the absence of the TF Pax6, but the explicit function of this tissue however is not yet understood (Assimacopoulos et al. 2003). The third dorsal signalling centre in the telencephalon, the anterior neural ridge is a prominent source of FGF proteins that have been found to be involved in DV as well as AP patterning. FGF8 for example is capable of inducing anterior fate by suppressing Wnt expression when ectopically expressed in posterior cortical regions (Fukuchi-Shimogori and Grove 2001). However FGF mouse mutants suggest that these growth factors act to coordinate the function of the different signalling centres in the telencephalon, because they show defects in BMP, Wnt and SHH signalling (Storm et al. 2006, for review see: Rash and Grove 2007).

In general, it is believed that cell signalling can act via gradients to control gene expression of responsive cells, by distinct intracellular pathways that conduct the extrinsic signal (Pires-daSilva and Sommer 2003; Tannahill et al. 2005). Although BMP Wnt and SHH signalling are transduced intracellularly by distinct proteins they all involve membrane receptor activation, phosporylation, the nuclear translocation of TFs, and their activation (for review see: Nusse 2003; Liu and Niswander 2005; Clevers 2006)

Cortical neurogenesis

During a period of approximately 8 days the mouse cerebral cortex develops from the neural tube, a single layer of epithelia-like precursor cells, into its 6 layered structure. During this time, precursor cells proliferate extensively and undergo profound changes, before neuronal differentiation and migration starts to generate the complex cellular organisation of the cerebral cortex (Casanova and Trippe 2006).

Cortical precursor cells: neuroepithelial cells, radial glial cells and intermediate progenitors

The neuroepithelial (NE) cells are of elongated shape and contact the basement membrane as well as the ventricular lumen where they attach to their neighbouring cells by adherens

junctions (AJs), tight junctions (TJs) and gap junctions (Shoukimas and Hinds 1978; Astrom and Webster 1991; Aaku-Saraste et al. 1996; Götz and Huttner 2005). The neuroepithelium, although a single cell layer, appears stratified as the nuclei of NE cells migrate along the apico-basal axis. This interkinetic nuclear migration (INM) is coordinated according to the cell cycle, such that S-phase occurs when the nucleus is at basal positions and mitosis when the nucleus is at apical positions (Takahashi et al. 1996; Murciano et al. 2002). Recent data propose that INM indeed depends on the proceeding cell cycle and the regulation of centrosome-associated microtubles (Ueno et al. 2006; Xie et al. 2007). NE precursor cells are the earliest neural precursor cells and proliferate symmetrically to expand their own population. Later on they give rise to neurons and RG cells that in turn generate not only neurons but also glial cells later in development. Neurons derived from NE cells are the first that are generated in the developing cortex and are destined to settle in the preplate (Casanova and Trippe 2006; Pinto and Götz 2007). With the onset of neurogenesis, NE cells start to generate RG cells that continuously replace the former. RG cells share certain aspects of NE cells but also show several glial properties. Like NE precursor cells, RG cells are highly polarized, connected to the basement membrane basally and to their neighbouring cells apically, where they form cell-cell junctions (Shoukimas and Hinds 1978; Mollgard et al. 1987; Astrom and Webster 1991; Aaku-Saraste et al. 1996). In terms of gene expression, NE and RG cells share the intermediate filament Nestin and its derivative RC2 but only RG cells express glial genes as S100β, brain lipid binding protein (BLBP) or the astrocyte specific glutamate transporter GLAST (Götz and Huttner 2005; Pinto and Götz 2007). RG cells undergo INM as NE cells do, however in contrast to NE cells their cell somata remain below the arising cortical neuronal layers, in the ventricular zone and only extends a thin basal process towards the basement membrane (Götz and Huttner 2005). Replacing NE cells as the predominant precursor cell type of the cerebral cortex with the onset of neurogenesis, RG cells self-renew to further expand the pool of neural progenitor cells and generate all remaining neurons originating from the developing cortex (Malatesta et al. 2003). This occurs either directly, or via the formation of an intermediate progenitor (basal progenitor) cell. Whereas the self-renewal of RG cells has been observed by symmetric as well as asymmetric cell division, the generation of neurons and intermediate progenitor (IP) cells so far has only been observed in asymmetric divisions (Miyata et al. 2004; Noctor et al. 2004; for review see: Huttner and Kosodo 2005). Upon their generation by apical mitosis IP cells migrate upwards to undergo S-phase in basal positions, retract their apical process and lose their contact to the ventricular lumen (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). IP cells

were originally discovered as a distinct precursor population of the developing cortex as they reside and divide at basal positions superficial to the ventricular zone. Therefore these progenitor cells are also referred to as subventricular zone (SVZ) progenitors. As IP cells lack both apical and basal processes, they also do not exhibit INM (Götz and Huttner 2005). These progenitors are characterized by a specific gene expression pattern, as they express Cux2, the non-coding RNA Svet1 and Tbr2 but not Pax6 that is expressed by NE and RG cells (Tarabykin et al. 2001; Nieto et al. 2004; Zimmer et al. 2004; Englund et al. 2005). Furthermore IP cells, in contrast to NE and RG cells self-renew less frequently and predominantly divide symmetrically to generate two neurons (Haubensak et al. 2004; Wu et al. 2005). Thus neurons generated within the dorsal telencephalon originate mainly from three different precursor populations, NE cells, RG cells and IP cells.

Cortical neurons forming the 6 layers of the cerebral cortex

The mammalian cerebral cortex displays a characteristic compared to other regions of the CNS, as it acquires a highly complex structure consisting of 6 neuronal layers in the neocortex and 3 in the archicortex. This layered architecture arises continuously with development starting from the preplate formed by the earliest neurons generated from NE cells around embryonic day (E) 11 (Casanova and Trippe 2006). By E12, newly generated neurons migrate into the preplate splitting it into the superficial marginal zone and the deep layered subplate. Subsequently generated neurons form the cortical plate in-between the marginal zone and the subplate. The laminar architecture of the cortical plate arises in an inside-out manner with the latest generated neurons forming the most superficial layers. This pattern is achieved as new born neurons migrate out of the ventricular or subventricular zone, either by somal translocation or by migrating along the processes of RG cells. By this mechanism they cross the already established cortical layers and settle above them, but beneath the marginal zone (Casanova and Trippe 2006; Molnar et al. 2006). The different cortical layers can not only be distinguished by their individual cytoarchitecture but also by the specific gene expression pattern of their neurons. Layer I mostly consists of Cajal-Retzius cells expressing the extra-cellular matrix protein Reelin and / or Calretinin. Upper layer neurons that from layer II and III are characterized by their expression of Cux1, 2, Brn1, 2 or Svet1 whereas neurons of layer IV and V can be distinguished from layer VI neurons as the former express ER81 whereas the latter are positive for Tbr1 and Foxp2 (Molyneaux et al. 2007). This individual patterning is already established by the end of neurogenesis at the day of birth and further sharpens with maturation. Those cortical neurons that are generated by

dorsal telencephalic precursor cells are excitatory cortical projection neurons and use glutamate for neuronal transmission. In contrast, cortical inhibitory interneurons are mostly generated from ventral telencephalic precursors and migrate tangentially into the developing cortex (Götz and Sommer 2005). Early generated interneurons integrate mostly into the preplate whereas later generated interneurons migrate along the intermediate zone and the SVZ to settle adjacent to cortical projection neurons that had been generated concurrently with them. Thus distinct cortical layers are formed by gluatmatergic projection neurons and GABA-ergic interneurons that have been generated during the same period (Casanova and Trippe 2006).

Extrinsic and intrinsic cues control cortical neurogenesis

Proliferation of precursor cells

The formation of an organ as complex as the cerebral cortex, requires tight regulation of proliferation, specification and differentiation of precursor cells and their progeny. During the early phases of cortical development NE cells divide within their epithelial plane expanding the neuroepithelium laterally. From the onset of neurogenesis the cerebral cortex expands radially by the generation of neurons and IP cells (Huttner and Kosodo 2005). The exact number of precursor cells present at the onset of neurogenesis and the precise timing of the switch from purely proliferative to differentiating cell divisions is essential to regulate cortical size and architecture (Guillemot et al. 2006). An ectopic increase in the number of precursor cells, achieved by reduction of apoptosis or cell cycle exit causes a substantial increase in cortical size. Inhibition of apoptosis, for example, by targeting Caspase 9 dependent activation of Caspase3 lead to an unorganized overgrowth of cortical tissue (Cecconi et al. 1998; Kuida et al. 1998). Similarly, blocking cell cycle exit by over-expressing constitutively active β-catenin considerably increased cortical size (Chenn and Walsh 2002; Chenn and Walsh 2003). Conversely, extensive exit from the cell cycle upon the deletion of Notch signalling rapidly depleted the progenitor pool, as observed in the Hes1, 3 and 5 triple mutant (Hatakeyama et al. 2004). Not only external signalling but also intrinsic cues have been found to influence proliferation in cortical progenitor cells. Deletion of the mouse homolog of Drosophila lethal giant larvae (Lgl1), for example, caused reduced cell cycle exit in the ventral telencephalon (Klezovitch et al. 2004) leading to an overgrowth of the GE. Conversely, the knock down of Par3, involved in apical positioning of the Par complex leads to the immediate terminal differentiation of cortical precursor cells *in vitro* (Costa et al. 2007). Interestingly, regulation

of mitotic spindle orientation was also observed to control cortical size in mouse and in human. The lack of the Lis1-interacting protein Nde1 was shown to cause delayed mitotic progression due to defects in spindle orientation and assembly as well as chromosome localization (Feng and Walsh 2004). This reduced the pool of progenitor cells in the developing cortex, such that specifically neurons of superficial cortical layers were decreased in number. Another molecule, the mammalian ortholog of Drosophila abnormal spindle gene (asp) ASPM, has been shown to control cleavage plane orientation, thereby influencing asymmetric cell division (Fish et al. 2006). The loss of ASPM leads to a reduced number of NE cells in mouse (Fish et al. 2006) and was found to be the most common cause of microcephaly in humans (Bond et al. 2002). Thus proliferation and therefore self-renewal of cortical precursor cells is tightly controlled by a variety of mechanisms that may act in parallel and interdependent of each other.

With the onset of neurogenesis, cortical precursor cells appear in the cortex that divide asymmetrically to regenerate themselves and to give rise to a neuron or IP cells (Miyata et al. 2001; Noctor et al. 2001; Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Such neurogenic precursor cells were, for example, identified by studies using transgenic mice expressing GFP either under the promotor of Tis21 or α-tubulin (Sawamoto et al. 2001; Haubensak et al. 2004), however it is unclear whether both demark the same precursor population. Notably mechanisms that have been shown to control precursor proliferation have also been found to influence IP generation. Notch signalling, for example, promotes RG cell proliferation and concurrently inhibits the generation of IPs although by distinct mechanisms, as the latter is dependent on the canonical Notch effector c-promotor binding factor1 (CBF1), while the former is not (Mizutani et al. 2007). In this regard it is noteworthy that negative interference with Notch signalling by the deletion of Presenilin1, causing precocious neurogenesis, also increased the cell cycle length of cortical precursor cells (Yuasa et al. 2002). This is in line with observations showing that precursor cells committed to a neurogenic fate, identified by Tis21-GFP, display a longer cell cycle compared to non-neurogenic precursor cells (Calegari et al. 2005). In addition to the short-range signalling of Notch between adjacent cells, canonical Wnt signalling, considered as a long-range signal, has been recently implicated in controlling the formation of IP from RG cells. Expression of constitutively active β-catenin in RG cells was reported to delay IP generation by promoting RG fate and subsequently delay the generation of upper layer neurons (Wrobel et al. 2007). Besides these extrinsic factors, intrinsic cell signalling pathways have been implicated in the generation of IP cells from RG cells. The small GTPase Cdc42, participating in the activation

of the Par-complex, is required to maintain RG fate, likely at the expense of IP cell generation (Cappello et al. 2006). This was apparent from Cdc42 loss of function experiments showing that RG cells detach from the apical surface and down-regulate the expression of Pax6. Conversely, the number of Tbr2 positive cells was increased in Cdc42 deficient cortices (Cappello et al. 2006). Thus, extrinsic as well as intrinsic signalling mechanisms regulate precursor proliferation and control the progression from a self-renewing RG to a further restricted neuronal precursor.

Neuronal commitment, differentiation and maturation

RG and IP cells both generate neurons and are committed to neuronal fate by the progressive expression of the proneural genes Neurogenin1 (Ngn1), Neurogenin2 (Ngn2) and Mash1 (Fode et al. 2000; Nieto et al. 2004). Ngn1, similar to Ngn2, functions as a TF to promote neuronal fate, but in addition inhibits astrogliogenesis in a manner independent of its DNA binding capacities (Sun et al. 2001). It does so by interfering with BMP and STAT signalling that act together to trigger the expression of the astrocytic marker GFAP. Ngn1 and 2 are highly redundant and both promote the expression of neuronal differentiation genes such as NeuroD1, NeuroD2, Math2, Math3 and Nscl1 (Schuurmans et al. 2004) that are sequentially expressed during neuronal differentiation and may be involved in its regulation (Guillemot et al. 2006). However Ngn1 and 2 are only required for the generation of early generated types of neurons committed for deep cortical layers whereas upper layer neurons have been shown to rely on the expression of Pax6 (Tarabykin et al. 2001; Schuurmans and Guillemot 2002; Schuurmans et al. 2004; Zimmer et al. 2004). Whereas Ngn1/2 double mutants lack deep layer neurons but display upper layer neurons, the Pax6 deficient cortex lacks the latter but has relatively little defects in neurons of layer V and VI. Despite the partially detailed knowledge of neuronal commitment and the molecular interplay of different proneural genes, the signalling pathways controlling the expression of these genes are not clearly identified (Campbell 2005; Guillemot et al. 2006; Takahashi and Liu 2006).

It has been suggested that cortical precursor cells follow an intrinsic differentiation program rather than being guided by extrinsic cues, which they receive from their environment. This notion was raised by the observation that different neuronal subtypes could be sequentially generated from cortical precursor cells *in vitro* (Qian et al. 1998; Qian et al. 2000; Shen et al. 2006). However transplantation studies demonstrated that early cortical precursor cells, determined to generate deep layer neurons, switch to the generation of upper layer neurons when transplanted into a developmentally more progressed environment (McConnell 1988;

McConnell and Kaznowski 1991). Further analyses revealed that precursor cells lose this capability with the onset of S-Phase and that their laminar fate potential gets progressively restricted with development (Frantz and McConnell 1996; Desai and McConnell 2000).

Once postmitotic neurons are generated in the VZ or SVZ they start to migrate out of the germinal layers to reach their appropriate laminar positions. *In vivo* imaging studies lead to a model of 4 distinct phases during neuronal migration: (1) the neuron proceeds to the SVZ where it (2) sojourns for 24h displaying multiple processes, then (3) the neuron transiently moves retrogradely extending a process towards the apical surface that later forms the axon, before (4) it finally migrates basally into the cortical plate (Tabata and Nakajima 2003; Noctor et al. 2004). Within the cortical plate newly arrived neurons settle on top of the already established cortical layers. Therefore they have to cross the already established cortical layers and do this by migrating along and in close contact to the radial glial fibres. This interaction has been implicated in the regulation of neuronal migration by signalling molecules such as Reelin. Studies on Reelin deficient "Reeler mutants" showed that this molecule is required for the splitting of the preplate and the inside-out organisation of the cortical layers (for review see: Goffinet 1979; Caviness 1982; Lambert de Rouvroit and Goffinet 1998). Reelin is secreted by Cajal-Retzius cells of the marginal zone and supposedly acts on RG cells rather than neurons as these cells express both the Reelin receptors VDLDR and ApoER2 as well as the intracellular effector Dab1 (D'Arcangelo et al. 1999; Hartfuss et al. 2003; Hack et al. 2007). This notion is also supported by the observation that the RG cells in the Reeler cortex display an abnormal morphology and convert prematurely into astrocytes (Forster et al. 2002; Hartfuss et al. 2003). Finally, upon establishing themselves in their appropriate layer, cortical pyramidal neurons develop axonal projections dependent on their identity and location. Neurons of layer II/III project to the brainstem and spinal cord, whereas deep layer neurons predominantly project through the corpus callosum into the contralateral hemisphere (Price et al. 2006). Several signalling pathways are known that guide axons along their pathways by acting as attractive or repulsive cues (Lindwall et al. 2007). Long-range diffusible factors like Robo and Slit but also morphogens like SHH and FGFs have been shown to play a significant role in axon guidance and the formation of cortical commissures, where cortical axons cross the midline to innervate the contralateral hemisphere. Recently Wnt ligands have been reported to participate in this process, as Frizzled3 mutant mice lack major axon tracts (Wang et al. 2006) and Wnt5a can repel cortical axons dependent on the developmental stage (Keeble et al. 2006).

Individual developmental processes as proliferation, differentiation or maturation are regulated by different mechanisms including long- and short range extrinsic signals but also intrinsic signalling cascades, suggesting that a tight and complex control is needed to establish complex neuronal networks. Conversely, single signalling pathways do not only regulated one distinct developmental process but influence various processes at different developmental stages by even acting through distinct mechanisms.

Wnt signalling

Such a key signalling pathway, participating in a variety of developmental processes is exerted by Wnt ligands and their receptors. This signalling pathway is evolutionarily conserved and found in all so far investigated animal species (Pires-daSilva and Sommer 2003). Wnt molecules are cysteine rich proteins that become post-translationally modified by glycosylation as well as palmitoylation. Whereas the first appears to be involved in extracellular movement of Wnt molecules, the lipid modifications are required for correct secretion as well as signalling activity (Hausmann et al. 2007). So far more than 20 Wnt proteins have been found in mammals that can be grouped into 12 subfamilies. Interestingly not all Wnt ligands signal through the same intracellular cascade, with some even activating different cascades during different developmental processes (Clevers 2006). Several Wnt ligands have been shown to be expressed in various parts of the developing CNS with Wnt2b, 3a, 5a, 7a, 7b and 8b expressed in the dorsal telencephalon (Parr et al. 1993; Grove et al. 1998; Lako et al. 1998). Of these Wnt ligands Wnt 2b, 3a, 7b and 8b have been reported to act through the canonical signalling cascade involving β-catenin (Galceran et al. 2001; Wang et al. 2005; Cho and Cepko 2006; Lee et al. 2006) whereas Wnt5a and Wnt7a have been reported to act via non-canonical pathways as well (Dabdoub et al. 2003; Adamska et al. 2005; Mikels and Nusse 2006; Nemeth et al. 2007). This difference is believed to be achieved at least partially by specific Wnt receptors activating distinct downstream effector cascades (Mikels and Nusse 2006). The main receptor type bound by Wnt ligands are seven-transmembrane-spanning proteins of the Frizzled (Fz) family. To transduce specifically canonical Wnt signalling they interact with low-density-lipoprotein-related-proteins (LRP) (Cadigan and Liu 2006). The family of Fz receptors contains a similar variety of proteins as the Wnt ligand family, several of which are expressed in the dorsal telencephalon, in spatio-temporal dynamic patterns (Kim et al. 2001). In contrast only 2 LRPs are known that function as Wnt co-receptors, LRP5 and 6, both expressed in the dorsal telencephalon (He et al. 2004; Zhou et al. 2006). Although the demonstration of a direct Fz-LRP interaction is still lacking

(Cadigan and Liu 2006), forced association of both molecules induced Wnt signalling activity *in vitro* and Fz8 was shown to bind LRP6 in a Wnt dependent manner, suggesting that association of both receptors occurs upon the binding of Wnt ligands (Tamai et al. 2000; He et al. 2004; Cadigan and Liu 2006). As the first step of signal transduction, the activation of Fz receptors and LRPs by Wnt ligands leads to the recruitment of the intracellular molecule dishevelled (Dvl) to the plasma membrane (Cadigan and Liu 2006). Fz receptors have only recently been discovered to be G-protein coupled and it is now known that G-protein activation is required to transduce Fz receptor function in both, canonical as well as non-canonical pathways (Quaiser et al. 2006). Also Dvl takes part in the canonical as well as in non-canonical pathways. The exact signal transduction cascade following Wnt ligand binding is still not fully understood although increasingly more proteins are found to participate in it (www.stanford.edu/~rnusse/wntwindow.html). The canonical Wnt signalling pathway finally leads to the disruption of a cytoplasmic protein complex that is mainly formed by the tumor suppressor protein adenomatous polyposis coli (APC), the Glycogen-synthase-kinase3β (Gsk3β) and Axin that serves to connect the other two. This complex clusters β-catenin that is subsequently phosphorylated by Gsk3β and by this finally primed for degradation through the proteasome. Disruption of the APC complex by Wnt signalling, probably through recruitment of Axin via Dvl to the plasma membrane, leads to the release of β-catenin from this complex, preventing its degradation and permitting the translocation of β-catenin from the cytoplasm to the nucleus (Behrens et al. 1998; Huelsken and Birchmeier 2001; Cadigan and Liu 2006) where it acts as a transcriptional activator (see below).

In addition to the canonical Wnt signalling, a second signalling cascade can be activated by Fz/LRP via Dvl, the planar polarity pathway (Seifert and Mlodzik 2007). This pathway involves the activation of Rho-kinases and controls mostly cell morphological changes and therefore plays important roles in epithelial rearrangements including convergent extension or neurulation. The planar polarity pathway has been reported to be activated by Wnt11, 5 and 7a. Fz receptors can also act independently of LRPs, this has been observed in the Wnt/Ca^{2+} pathway inducing intracellular Ca^{2+} release and the subsequent activation of CamKII (Huelsken and Behrens 2002). Moreover, the time and region specific expression of various Wnt signalling inhibitors displays another tool to increase Wnt signalling diversity. Fz related molecules, that share the cystein rich Wnt binding domain of Fz receptors but lack their transmembrane domain, are secreted inhibitors that sequester Wnt ligands extra-cellularly (Kawano and Kypta 2003). In contrast, secreted Dickkopf (Dkk) proteins inhibit specifically

LRP5/6 as their binding induces the rapid endocytosis of LRP5/6 (He et al. 2004). By this they inhibit specifically β-catenin dependent canonical Wnt signalling.

β-catenin dependent Wnt signalling in neurogensis

β-catenin, when freed from the APC complex is translocated to the nucleus where it acts together with T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins as a transcriptional activator (Huelsken and Behrens 2002). In the absence of Wnt and thus of β-catenin from the nucleus, TCF/LEF proteins function as a transcriptional repressor. Through their HMG domain TCF/LEF proteins bind to a conserved DNA sequence, the Wnt response element, where they interact with co-repressors such as CtbP or Groucho/TLE (Willert and Jones 2006; Hoppler and Kavanagh 2007) preventing transcription of Wnt signalling target genes. The binding of β-catenin removes the co-repressor proteins and recruits instead co-activator complexes such as CBP/p300 or the chromatin remodelling complex SWI/SNF (Bienz and Clevers 2003). SWI/SNF contains the ATPase Brg1 that interacts with β-catenin to bend the chromatin surrounding the TCF binding region and enhances TCF-responsive gene transcription (Barker et al. 2001). Prominent target genes of canonical Wnt signalling are CyclinD1, Sox2, Sox9 and c-myc, that are predominantly involved in cell proliferation. Also genes involved in Wnt signalling regulation such as Axin2 (Conductin), TCF1 and LEF1 have been shown to be regulated by β-catenin dependent Wnt signalling (www.stanford.edu/~rnusse/wntwindow.html). During CNS development, canonical Wnt signalling does not only control precursor cell proliferation but has been shown to influence regional patterning (Backman et al. 2005) neuronal differentiation (Hirabayashi et al. 2004; Prakash and Wurst 2007), axonal guidance (Lindwall et al. 2007) and synaptogenesis (Packard et al. 2003; Davis and Ghosh 2007). In the developing dorsal telencephalon Wnt ligands are mostly expressed at medial regions with Wnt2b, 3a and 5a expressed exclusively in the cortical hem and Wnt8b expanding from the hem into the anlage of the hippocampus. Within the developing neocortex only Wnt7a and 7b are expressed, with the former restricted to germinal layers and the latter expressed in neurons (Grove et al. 1998; Rubenstein et al. 1999). The data obtained from Wnt3a knockout mice revealed that Wnt signalling in the dorsal telencephalon is crucial for the expansion of hippocampal progenitor cells and neural crest development (Lee et al. 2000). However Wnt ligands overlap in their expression domains and Wnt1/3 double mutants revealed a more severe phenotype compared to the respective single mutants, strongly suggesting that Wnt ligands can act in a redundant manner, substituting for each other (Ikeya et al. 1997). To circumvent this, LEF (Galceran et al. 2000)

and β-catenin mutants (Haegel et al. 1995; Huelsken et al. 2000) were generated to gain more comprehensive information about the role of Wnt signalling. As β-catenin mutants die very early during development (Haegel et al. 1995; Huelsken et al. 2000), conditional ablation of β-catenin was further on used to investigate canonical Wnt signalling during CNS development (Brault et al. 2001). Loss of function experiments deleting β-catenin within the hind and midbrain revealed that canonical Wnt signalling is essential for the development of these regions as its absence resulted in increased apoptosis and cell cycle exit (Brault et al. 2001; Zechner et al. 2003). D6-Cre mediated deletion of β-catenin abolished canonical Wnt signalling in the hippocampal anlage and the dorso-medial cortex (Machon et al. 2003), confirming the essential function of canonical Wnt signalling in the formation of the hippocampus that have been apparent from Wnt1/3 knockout experiments (Ikeya et al. 1997; Lee et al. 2000). Recent experiments expanded the understanding of the role of canonical Wnt signalling in hippocampus development, as they demonstrated that the remainingly high levels of Wnt expression in the medial cortex during development induce the specification of hippocampal neuronal subtypes (Machon et al. 2007). The loss of function studies on β-catenin dependent Wnt signalling were complemented by experiments in which canonical Wnt signalling was ectopically activated, specifically in precursor cells of the CNS. A constitutively active form of β-catenin, lacking the phosphorylation domain for GSK3β, was expressed under a Nestin enhancer element, demonstrating that ongoing canonical Wnt signalling keeps precursor cells within the cell cycle and prevents them from generating neurons. β-catenin over-expressing brains were greatly enlarged and showed gyrus-like undulations in the dorsal telencephalon (Chenn and Walsh 2002; Chenn and Walsh 2003). Interestingly although the β-catenin loss- and gain-of-function experiments showed consistent malformations, the mechanisms reported to cause these phenotypes varied dependent on the strategies used. Over-expression of β-catenin in the entire CNS (Chenn and Walsh 2002) and its deletion in the mid- and hindbrain were reported to affect cell cycle exit in reciprocal manners (Brault et al. 2001; Zechner et al. 2003), whereas Machon et al 2003 did not observe differences in cell cycle exit upon deletion of β-catenin in the dorsal telencephalon. Similarly, changes in apoptosis were reported by two studies (Brault et al. 2001; Zechner et al. 2003) but not by others (Chenn and Walsh 2003; Machon et al. 2003), suggesting that β-catenin serves cell type specific functions even within the precursor populations of the CNS. This notion was further supported by the finding that canonical Wnt signalling regulates dorso-ventral patterning of the telencephalon during a narrow time window that finishes with the onset of neurogenesis (Backman et al. 2005). Following patterning, canonical Wnt signalling seems to

predominantly regulate the expansion of the precursor pool before it displays again distinct functions during neuronal maturation, axonal pathfinding and synaptogenesis (Davis and Ghosh 2007; Lindwall et al. 2007). In the adult stem cell niche of the hippocampus, however canonical Wnt signalling maintains its proliferative role. Secreted from astrocytes in the dentate gyrus, Wnt3a specifically enhances the proliferation of neuroblasts and induces their neuronal fate (Lie et al. 2005).

β-catenin dependent Wnt signalling in tumor formation

The dominant proliferative potential of canonical Wnt signalling promotes not only extensive proliferation of the neural tissue but has also been observed in other organs and during their development (Clevers 2006). After organogenesis is finished, proliferative events come to a hold and are further restricted to distinct targets as for example adult stem cell niches. Ectopic activation of β-catenin dependent Wnt signalling often leads to overproliferation and cancer formation. Interestingly most cancers caused by over-activation of canonical Wnt signalling are induced by mutations of genes participating in the β-catenin destruction complex. Most prominently the tumor suppressor gene APC was one of the first mutations characterized in tumor formation. A defective APC allele is inherited in the Familial Adenomatous Polyposis cancer syndrome causing the formation of numerous colon adenomas and malignant adenocarcinomas during early adulthood (Clevers 2006). Mutations of Axin or β-catenin are less frequent. However all of these mutations lead to the inappropriate maintenance of β-catenin, activating β-catenin/TCF-LEF controlled gene transcription.
Interestingly malignancies of tumors is negatively influenced not only by extensive proliferation and abolished apoptosis but also by increased cell motility, aiding tissue invasion and spreading of carcinogenic cells. The motility of cells is mostly inhibited by inter-cellular adhesion keeping them positioned within their tissue. And again β-catenin plays a major role in this mechanism connecting tightly proliferation and cellular adhesion, two processes that are important not only in cancer formation but also during embryonic development.

Cadherin based inter-cellular adhesion

Cell adhesion is fundamental to tissue and organ development as it enables the three dimensional organisation of single cells into a multi cellular organism. Adhesion can occur between individual cells as well as to an extracellular matrix (ECM). Different cell adhesion mechanisms have been identified, of which the most prominent may be Cadherin based

adhesion of epithelial cells (Gumbiner 1996; Schock and Perrimon 2002; Takeichi 2007). NE cells establish Cadherin based cell-cell contacts to neighbouring cells including tight- and adherens junctions (Astrom and Webster 1991; Aaku-Saraste et al. 1996). RG cells in contrast have been reported to lack TJs and only form AJs (Aaku-Saraste et al. 1996). In sheep cortex however TJs like cell-cell contacts were reported, that did not arrange in a belt-like fashion and were localized in some distance from the ventricular surface (Mollgard et al. 1987). In addition, cell-cell junctions have been observed in the mouse cortex at E13/E15 that shared the appearance of zonulea adherents but displayed properties of tight junctions as well as Gap junctions (Shoukimas and Hinds 1978). Thus, RG cells as NE cells are apically connected to their neighbouring cells but might display a more dynamic or polymorphic interaction.

AJs are based on classical Cadherin adhesion receptors that form homophilic complexes with their extra-cellular domains dependent on the presence of Ca^{2+}, thereby establishing cell-cell contacts (Gumbiner 2000). Intracellularly Cadherins exhibit two different domains binding β-catenin and p120 catenin respectively (Gumbiner 1996; Castano et al. 2002). The formation of AJs develops from primordial contacts, so-called puncta, through the establishment of cell-cell contacts lateral to the puncta. Finally these contacts mature and expand through the further clustering of Cadherin molecules (Perez-Moreno and Fuchs 2006). By interactions between β-catenin and α-catenin, Cadherin clusters in AJs become connected to the F-actin cytoskeleton. This interaction has been suggested to be very dynamic as it was demonstrated that α-catenin binds to β-catenin as a monomer whereas it binds to F-actin in a dimeric state (Drees et al. 2005; Yamada et al. 2005). In addition to this core complex, existing of Cadherins, β-catenin and α-catenin, an extremely diverse group of molecules has been found to interact with AJs or localize adjacent to these structures. Two other adhesion molecules, Vinculin and ZO1, can bind to α-catenin (Watabe-Uchida et al. 1998; Imamura et al. 1999), which has also been reported to interact with actin remodelling molecules such as Arp2/3 (Verma et al. 2003) or Ena/Vasp (Scott et al. 2006). Small GTPases such as RhoA, Rac1 and Cdc42 were suggested to participate in AJ organisation and remodelling by interacting with the actin-cytoskeleton (Perez-Moreno and Fuchs 2006). Also molecules that are known to participate in cellular polarity such as Par3, Par6, Cdc42 and Numb, are localized in close proximity to the AJs (Manabe et al. 2002; Cappello et al. 2006; Rasin et al. 2007). In addition β-catenin directly participates in both adhesion and Wnt signalling and may thus directly contribute to the regulation of adhesiveness. This was suggested from experiments showing that conformational changes of β-catenin can favour association either with transcriptional or adhesive complexes (Harris and Peifer 2005).

Adherens junctions in cortical development

Most Cadherins are expressed in the CNS, participating in the formation of nuclear groups of neurons, in the formation of fibre tracts, in synaptogenesis, cell sorting and layer formation (Shapiro et al. 2007) but also in the cellular adhesion of cortical precursor cells (Redies and Takeichi 1996). R-Cadherin and Cadherin6 have been found to function in cell sorting and the formation of the pallial-subpallial boundary in a manner dependent on Pax6 (Stoykova et al. 1997; Inoue et al. 2001). Whereas epithelial (E) Cadherin and neural (N) Cadherin participate in the formation of cortical AJs between NE cells as well as RG cells (Redies and Takeichi 1996). IP cells in contrast delaminate from the epithelial sheet and do not exhibit AJs (Götz and Huttner 2005). The high redundancy between different Cadherin proteins might explain why the deletion of N-Cadherin in the developing cortex did not completely abolish AJs from the tissue (Kadowaki et al. 2007). However cortical organisation was disrupted in this mouse mutant, with neurons and precursor cells intermingling and impaired formation of the cortical layers. Similar phenotypes were observed in the absence of β-catenin, although these mutants exhibited additionally differentiation defects that hampered the interpretation in regard to the function of AJs (Brault et al. 2001; Machon et al. 2003; Zechner et al. 2003). Very early during cortical development β-catenin was reported to function only in cell adhesion as canonical Wnt signalling was claimed to be absent from NE cells (Junghans et al. 2005). At these early stages (prior to E10) the loss of β-catenin lead to a massive cell death suggesting that cortical tissue integrity rely more on AJs during early than later development. Similar to β-catenin, the deletion of α-catenin from neural precursor cells was reported to dissolve AJs. In addition the lack of α-catenin throughout the CNS resulted in the ectopic expression of SHH target genes, leading to the suggestion that cell-cell contact by AJs opposes proliferation, by inhibiting SHH signalling (Lien et al. 2006a). Despite the distinct aberrations in cell signalling all these mutants share the appearance of cortical disorganisation and the loss of distinct germinal and neuronal layers. Interestingly a very similar phenotype was observed upon deletion of Numb and Numb-like (Numbl), two highly redundant molecules, that are thought to be involved in Cadherin trafficking and recycling (Rasin et al. 2007).

Cellular adhesion and polarity

Interestingly Numb has also been implicated in cellular polarity (Knoblich et al. 1997), similar to other molecules that have been found to localize adjacent to AJs in cortical precursor cells. The Par complex, localised near AJs at the apical-lateral membrane of

epithelial cells, was reported to interact with the Numb complex (also including Crumbs) as well as with Gsk3β or Lgl (Suzuki and Ohno 2006). The diverse interactions of polarity molecules between each other but also with molecules involved in cell signalling complexes draws a very complex picture of cell polarity regulation. In this regard it is notable that deletion of Numb, together with Numbl, has been reported independently to cause opposing defects on cortical development (Li et al. 2003; Petersen et al. 2004). Whereas Li et al 2003 presented data that Numb and Numbl in the cortex are required to control timed cell cycle exit, Petersen at al 2004 observed increased differentiation after the loss of Numb and Numbl. In contrast, data obtained from loss of function studies on molecules of the Par complex in the dorsal telencephalon concurrently point to a function of this complex in maintaining precursor cells in a proliferative state. RG cells retract their apical processes upon the loss of Cdc42 or atypical protein kinase C λ (aPKC λ) and change towards differentiation (Cappello et al. 2006; Imai et al. 2006). Consistently Par3 becomes down-regulated in RG cells with proceeding neurogenesis and deletion of this molecule induces immediate cell cycle exit (Costa et al. 2007). However, Par3 is known to be required for Par complex localisation, rather than activity, raising the question whether impaired polar distribution might be sufficient to change the behaviour of cortical precursor cells.

Another molecule asymmetrically localized in NE and RG cells is Prominin (CD133) that has been found to be expressed in a broad variety of stem cells (Corbeil et al. 1998; Florek et al. 2005; Lee et al. 2005). In NE and RG cells Prominin is enriched on the microvilli of the apical membrane, reaching into the ventricular lumen (Weigmann et al. 1997). Although direct evidence for the exact role of Prominin in stem cells is still lacking, observations of symmetric and asymmetric cell division suggested a prominent role for this molecule. Neurogenic precursor cells, identified by Tis21 (see above) and thus considered to undergo asymmetric cell division, were shown to bisect the Prominin positive apical membrane in a manner that only one daughter cell inherits it. In contrast, non-neurogenic precursor cells bisect the apical membrane, inheriting Prominin to both daughter cells (Kosodo et al. 2004). Consistent with this, precursor cells reduce their amount of apical membrane before undergoing neurogenic divisions by releasing Prominin positive membrane particles into the cerebrospinal fluid (CSF) (Marzesco et al. 2005; Dubreuil et al. 2007). Precursor polarity, asymmetric cell division and neurogenesis appear thus tightly connected and are likely to ensure the precision with which the cerebral cortex is established during development.

Cell adhesion and polarity play key roles in the establishment and maintenance of the neuroepithelial sheet, in the delamination of cells and neuronal migration and therefore participate in the morphogenetic processes that lead to the formation of the 6 layered cerebral cortex. The number, fate and specification of the highly diverse cell types of the cortex are however controlled by extrinsic and intrinsic cell signalling mechanisms and gene transcription. One protein involved in both, cell signalling and adhesion is β-catenin that functions as the key transducer of canonical Wnt signalling but also in the formation of Cadherin based AJs between NE and RG cells. The aim of this work was to understand how β-catenin participates in the development of the cerebral cortex and how its functions vary in regard to developmental stages and specific cortical regions. To examine this I compared different conditional mutants, in which β-catenin was deleted from the telencephalon at different time points and in different regions. In addition, these mutants were compared to an conditional mutant lacking α-catenin, to reveal which of its functions β-catenin servers as a component of Wnt signalling and which as a component of the AJs.

Material and Methods

Animals

The mouse strains used in these work were:

(1) B6.129-Ctnnb1tm2Kem/KnwJ, carring floxed alles of β-catenin (Brault et al. 2001)
(2) B6;129-Ctnna1tm1Efu/J, carring floxed alles of α-catenin (Vasioukhin et al. 2001)
(3) Tg[Fos-lacZ]34Efu, expressing β-galactosidase under a TCF/LEF controlled promoter and used as a canonical Wnt reporter mouse line (DasGupta and Fuchs 1999)
(4) Emx1::Cre, expressing Cre Rekombinase in the Emx1 locus (Iwasato et al. 2000)
(5) hGFAP-Cre, expressing Cre Rekombinase from the human GFAP promoter (Zhuo et al. 2001)
(6) Nestin-Cre, expressing Cre Rekombinase under the control of a Nestin enhancer element (Tronche et al. 1999)

The B6.129-Ctnnb1tm2Kem/KnwJ, the B6;129-Ctnna1tm1Efu/J and the Tg[Fos-lacZ]34Efu mouse lines were obtained from Jackson Laboratory (http://www.jax.org/index.html). The Emx::1Cre line was kindly provided by Dr. Iwasato and Dr. Itohara, the hGFAP-Cre and Nestin-Cre lines were kindly provided by Dr. R. Klein, MPI für Neurobiology, München.

Genotyping of transgenic animals

Genotyping by poly chain reaction (PCR) was performed according to protocols provided by Jackson Laboratories for the B6.129-Ctnnb1tm2Kem/KnwJ, the B6;129-Ctnna1tm1Efu/J and the Tg[Fos-lacZ]34Efu stains.

For the other lines following DNA primers were used for genotyping:

Emx1::Cre: 5`-GTG AGT GCA TGT GCC AGG CTT G-3`
5`-TGG GGT GAG GAT AGT TGA GCG C-3`
5`-GCG GCA TAA CCA GTG AAA CAG C-3`

hGFAP-Cre: 5`-ACT CCT TCA TAA AGC CCT CG-3`
5`-ATC ACT CGT TGC ATC GAC CG-3`

Nestin-Cre: 5`-TTC GGA TCA TCA GCT ACA CC-3`
5`-AAC ATG CTT CAT CGT CGG-3`

Histology

Animals used in this work were mated 12-18h and the day of vaginal plug was considered as embryonic day (E) 0. Mice were anesthetised by Diethylether or CO_2 and sacrificed by cervical dislocation. Embryos were placed in Hanks Balanced Salt Solution (HBSS,Gibco) containing 10mM Hepes (Gibco) and immediately dissected. Brains or heads were fixed in 4% PFA, cryoprotected in 30% Succrose and cut in 12µm or 16µm thick section on a Cryostate (Leica).

BrdU labelling

To label acutely proliferating cells the DNA-base analogue 5'-bromo-2-deoxyuridine (BrdU, Sigma) dissolved in isotonic saline was injected intraperitoneally (5mg/100g bodyweight). For cell cycle exit analysis BrdU was injected at embryonic day (E) 10 and mice sacrificed 24h later, for analysis of the Labelling Index BrdU was administered at E11 and mice sacrificed 30 minutes later. For neuronal birthdating analyses BrdU was administered at E11, E12, E14 or E16 and animals analysed at the day of birth (P0).

Immuno-histochemistry

For immunolabelling cyrosections were, in general, incubated over night at 4°C in a solution containing the first antibody, 0,5%Triton 100 (Tx) and 10% normal goat serum (NGS).

Distinct pre-treatments were used to increase the specificity of different antibodys:
Citrate-buffer pre-treatment: sections were boiled 8 minutes in 0,01M sodium citrate (pH6) using a microwave.
Acid-ethanol pre-treatment: sections were incubated for 20 minutes in 20% (v/v) acetic acid in ethanol at -20°C
HCl-pre-treatment: sections were incubated in 2N HCl for 30 minutes and afterwards 2x 15 minutes in 0,1M Sodium-tetraborate (pH8,5)
Signal amplification by Thyramid-Kit: secondary antibody incubation was preceded by 30 minutes of incubation in 0,3%H_2O_2 in PBS and the secondary, biotinylated antibody was detected with Tyramide detection kit (Perkin Elmar Life Science).

F-actin was detected using Rhodamine labelled Phalloidin (1:40 in PBS) after incubation in 0,1%Tx (1h) and 10%NGS (20 minutes).

Secondary antibodies were applied in conditions according to the first antibody and incubated for 45-60 minutes at room-temperature.

Nuclei were visualized either by staining with 4.6-damindino-2-phenylindol (DAPI) (0,02mg/ml in H_2O) for 10 minutes or by propidium-Iodite (PI) (0,5mg/ml PI, 25U/ml RNAse in PBS).

Specimen were mounted with Aqua Poly-Mount and analysed using Confocal Microskopy (FV 1000, Olympus) or Lightmicroscopy (Axioplan2, Apoptome, Zeiss, Axiovision software 4.2) in combination with the Neurolucida Software 6.0 (MicroBrightField; Inc.,Vermont,USA).

First antibodies

recognized antigene	host-animal/Ig subtype	pre-treatment & incubating conditions*	obtained from
aPKCλι	mouse/IgG2b	used in WesternBlot	B.D.Transduction Lab.
BrdU	rat	HCL-treatment 1:100	Abcam
	mouse/IgG1	HCL-treatment 1:100	Bio-science products
Calretinin	rabbit	1:500	swant swiss antibodys
Caspase3	rabbit	1:50	Promega
Cre-recombinase	rabbit	1:50	Convance
GFAP	rabbit	Acid-ethanol treatment 1:500	Dako
Ki67	rat	Citrat-buffer treatment 1:50	Dako
Mash1	mouse/IgG1	Citrat-buffer treatment 1:2	Devel.Hybridoma Bank

Nestin	mouse/IgG1	1:3	Devel.Hybridoma Bank
pan-Cadherin	rabbit	Citrat-buffer treatment 1:200	Sigma
	mouse-IgG1	Citrat-buffer treatment 1:200	Sigma
Par3	rabbit	Citrat-buffer treatment 1:200	Upstate
Pax6	rabbit	Citrat-buffer treatment 1:500	Chemicon
	mouse/IgG1	Citrat-buffer treatment 1:20	Devel.Hybridoma Bank
phosphoryl. aPKCλι	rabbit	used in WesternBlot	B.D.Transduction Lab.
phosphoryl. Histone3	rabbit	1:200	Upstate Biotech
phosphoryl. MAPK	rabbit	1:100	Promega
Prominin	rat	1:500	eBioscience
Reelin	mouse/IgG1	1:10	Merk
Tbr1	rabbit	1:200	Abcam
Tbr2	rabbit	Citrat-buffer treatment HCL-treatment 1:500	Chemicon
α-catenin	mouse/IgG1	Citrat-buffer treatment 1:20	B.D.Transduction Lab.
β-catenin	mouse/IgG1	1h 1%Tx, 2,5%NGS 1:50, PBS, 2,5%NGS	B.D.Transduction Lab.
β-galactosidase	rabbit	1:50000 Tyramid-Kit	Capple
β-III-tubulin	mouse/IgG2b	Acid-ethanol treatment 1:100	Sigma

* if different from general descriptions

Secondary antibodies

Antibody	label	Supply
goat-α-rabbit	Cy2	Jackson Immuno Research
goat-α-rabbit	FITC	Jackson Immuno Research
goat-α-rabbit	Alexa546	Invitrogen
goat-α-rabbit	biotinylated	Vector Laboratories
goat-α-rat	Alexa488	Invitrogen
rabbit-α-rat	biotinylated	Vector Laboratories
goat-α-mouse IgG1	FITC	Southern Biotech
goat-α-mouse IgG1	Alexa546	Invitrogen
goat-α-mouse IgG2b	TRITC	Southern Biotech
goat-α-mouse IgG	Cy3	Jackson Immuno Research
Sterptavidin-Cy3		Jackson Immuno Research
Sterptavidin-Alexa488		Invitrogen

InSitu hybridisation

Plamid preparation:

Plasmids were chemically transformed into DH5α E.coli bacteria, these were plated onto LB-agar plates (15g agar/l) and incubated over night at 37°C. One of the grown colonies as picked and expanded in 100ml LB (20gLBbroth base/l) medium over night at 37°C. Specificity of plamid transformed bacteria was assured by Ampicilin selection. Plasmids were harvested using the Plamid Midi Kit (Qiagen), following the supplied protocol.

In vitro transcription:

20µg of plasmid were linearized with restriction enzymes, chosen according to the plasmid vector.

The linearized DNA was extracted by phenolchloroform and precipitated with 3M Sodium-acetate (1/10) and Isopropanol (70%v).

1µg of plasmid was used for invitro transcription by T3, T7 or SP6 RNA-polymerase.

1µg Plasmid

1mM ATP, 1mM CTP, 1mM GTP, 0,65mM UTP, 0,35mM DIG-11-UTP (Digoxigenin-RNA-labeling mix, Roche)

Transcription buffer, supplied together with the Polymerase

40U RNAse inhibitor (Invitrogen)

50U RNA-Polymerase (Invitrogen)

The reaction mix was incubated for 2h at 37° and the reaction was terminated by adding EDTA. RNA was purified using the RNeasy mini kit (Qiagen) following the supplied protocol.

Hybridisations of sections

Tissue sections were incubated in Hybridisation buffer [50%Formamid, 0,1%Tween, 10%Dextransulfate, 1mg/ml tRNA, 1xDenhard'ssolution in salt solution] containing 150ng RNA probe at 65°C over night in humid atmosphere. Subsequently sections were 3 times washed in washing solution [50%Formamid, 0,1%Tween in SSC] at 65°C and 2 times in MABT [100mM Maleic acid, 150mM NaCl, 0,02%Tween] at room-temperature. Sections were blocked in [2% blocking reagent, 20%NGS in MABT] for 1h and RNA probes were detected by Anti-Digoxigenin-AP Fab fragments (Roche) in [2% blocking reagent, 2%NGS in MABT] over night at room-temperature. Sections were washed 4 times with MABT and developed using NBT/BCIP (350µg,175µg/ml) in [100mM NaCl, 50mM $MgCl_2$, 100mM Tris (pH9,5), 0,1%Tween] over 1-3 days at 4°C.

Plamids:

Gene	Plasmid-vector	Restriction enzyme and RNA polymerase for antisense-probe	kindly provided by
BMP6	Bluescript SK(-)	SacI - T3	Dr.T.Theil Zoology Department, Trinity College Dublin, Ireland
conductin	Bluescript SK II+	XbaI – T7	Dr.B.Jerchow RCC, Roßdorf, Germany
Cux2	Bluescript SK	NotI – T3	Dr.C.Schuurmans IMCH, HBI, University of Calgary, Canada

Emx1	pGem3	EcoRI – Sp6	Dr.T.Theil Zoology Department, Trinity College Dublin, Ireland
Emx2	pGem - T	NcoI – Sp6	Dr.T.Theil Zoology Department, Trinity College Dublin, Ireland
Er81	Bluescript SK	Spe1 – T7	Dr.C.Schuurmans IMCH, HBI, University of Calgary, Canada
Erm		SalI – T7	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
FGF15		BamHI – T3	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
Gli1		NotI – T3	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
Hes-1	Bluescript SK	XhoI-T3	Dr.R.Kageyama Institute for Virus Research, Kyoto University,Japan
Hes-5	Bluescript SK	HindIII-T3	Dr.R.Kageyama Institute for Virus Research, Kyoto University,Japan
Id3		BamHI – T7	Dr.A.Mallamaci Department of Biological and Technological Research, Milan, Italy
Lhx2	CMV-mouse	HindIII – Sp6	
Lmx1a	pGEM-T	NdeI – T7	Dr.S.Retaux Institut de Neurobiologie Alfred Fessard, Centre National de la Recherche Scientifique, France.
Neuro D	Bluescript KS	KpnI – T7	Dr.C.Schuurmans IMCH, HBI, University of Calgary, Canada
Ngn2	Bluescript SK	SacI – T7	Dr.F.Guillemot National Institute for Medical Research, Mill Hill, UK
Patched1 (Ptc)	BluescriptII KS	BglII – T7	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
Pea		HindIII – T3	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany

Satb2	pGEM-T	Apa1 – Sp6	Dr.V.Tarabykin MPIem, Göttingen, Germany
Sef	pCS2	NcoI – Sp6	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
SVET1	pBS	Xhol – T7	Dr.P.Gruss Department of Molecular Cell Biology, MPI Biophysical Chemistry, Goettingen, Germany
Wnt 2b	pT7T3D-Pac	EcoRI – T3	Dr.E.Monuki Department of Pathology and Laboratory Medicine, University of California-Irvine, USA.
Wnt 3a	pGEM3Zf	ApaI – SP6	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany
Wnt 7a	Bluescript SK	XbaI – T7	Dr.A.McMahon Roche Institute of molecular biology, New Jesey, USA
Wnt 7b	Bluescript SK	ApaI – T3	Dr.A.McMahon Roche Institute of molecular biology, New Jesey, USA
Wnt 8b	pBSII KS	XbaI – T3	Dr.W.Wurst GSF – Institute for Developmental Genetics, Munich, Germany

Salt solution:

90mM NaCl, 10mM Tris base, 70mM NaH2PO4, 50mM Na2HPO4, 50mM EDTA in H2O

SSC (20x):

3M NaCl, 0,3M sodium citrate

Tunel staining

To detect apoptotic cells in tissue sections the "In Situ Cell Death Detection Kit,POD" (Roche) was used following the supplied protocol.

X-gal staining

β-galactosidase activity was detected by incubating tissue sections with 1mg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranose in [5mM$K_3Fe(CN)_6$, 5mM$K_4Fe(CN)_6$, 2mM$MgCl_2$]. To improve the reaction sections were pre-treated 30 minutes with [2mM MgCl2, 5mM EGT

in PBS] followed by 2x 15 min incubation in [2mM MgCl2, 0,01% sodiumdeoychore, 0,02%NP40].

DiI labelling of cortical cells

These experiments were done together with Leanne Godinho, PhD (GSF, Institute for Stem Cell Research, Munich)

E13 cortices were dissected in ice-cold HBSS/10mM HEPES. The meninges were carefully removed and the hemispheres were separated and incubated in 10mg/ml 1, 1'-dioctadecyl-3,3, 3',3'-tetramethylindocarbocyanineperchlorate (DiI) in HBSS for 15 minutes on ice. Following washes in HBSS, the hemispheres were embedded in 2.5% low melting agarose (Biozym Scientific) and 250 □m coronal slices were cut using a vibratome (Microm). Slices were recovered in ice-cold HBSS, transferred to a glass-bottom culture dish (Mat Tek), embedded in collagen (extracted from rat tail) and overlayed with Neurobasal medium supplemented with 10% heat-inactivated Foetal calf serum, 1xN2, 1xB27 and 100 units/ml penicillin/streptomycin (all from Gibco-Invitrogen). Following incubation in a tissue culture incubator, slices were fixed in 4% PFA for 1 hour.

Western Blot analysis

This experiment was performed together with Franziska Weinandy (GSF, Institute for Stem Cell Research, Munich).

Cortical tissue was harvested and lysed in RIPA buffer [20mM TRIS, 137mM NaCl, 10%Glycerol, 0.1%SDS, 0.5%Deoxycholate, 1%Triton100, 2.0mM EDTA, 10mM DTT, 20µg/ml Aprotinin, 1µg/ml Leupeptin, 1mM PMSF, 1mM Na-O-Vandat]. 20µg of protein was loaded on 10% SDS-gel followed Laemmli. Gels were blotted onto PVDF membranes (Biorad) and stained for α-catenin (1:250), Par3 (1:500), aPKC (1:250) and P-aPKC (1:500. Horseradish peroxidase labelled secondary antibodies (Amersham) were detected by using ECL Western Blotting Detection (Chemicon).

Electron Microscopy

These experiments were performed by Michaela Wilsch-Bräuninger, PhD (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden) and in the Fakulty of Medicine, Ludwigs Maximilans Universität München, in the *Insitut*e of Prof. Dr. Mehdi Shakibaei.

Conventional electron microscopy was performed as described in Cappello et al. (2006) after cutting 400 um vibratome sections (Leica Company) through E11 or E13 mouse brains.

Data analysis

Cortical size, number of PH3-positive and apoptotic cells were quantified using Neurolucida software 6.0 (MicroBrightField;Inc.,Vermont,USA). Cortical size was defined from the pallial-subpallial boundary to the medial cortical hem. The number of PH3 immunopositive cells and apoptotic cells were counted and calculated as the number of cells per μm^2. All other quantifications were done using single confocal optical sections. The Labeling Index was obtained by quantification of cells labeled by a 30 minute BrdU pulse, subtracting the number of neurons. Cell cycle exit was quantified by double-staining for BrdU and β-III-tubulin and counting the number of single and double positive cells within an area extending from the ventricle to the basal lamina. For all quantifications at least two different litters of animals were analysed.

Microarray analysis

These experiments were performed together with Martin Irmler PhD (GSF, Institute of Experimental Genetics, München)

Total RNA was isolated from cortical tissue of WT and α-cat-/- embryos at E11 and E13 using the RNeasy Mini kit (Qiagen) including DNase treatment. RNA quality was analysed using Agilent 2100 Bioanalyzer and Pico Labchip kit. RNA of WT and α-cat-/- animals from three different litters was amplified using the MessageAmp II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion) and hybridized on Affymetrix MOE430 2.0 arrays containing about 45k probe set. Staining and scanning was done according to the Affymetrix expression protocol. For statistical analysis of the expression data the Bioconductor software package implemented in Carma web (Rainer et al. 2006) was employed using RMA preprocessing and the paired moderated limma test. For the analysis of E11 samples the probe sets with the highest variance were used (40 % of all). The Benjamini-Hochberg algorithm was used to identify genes with a false discovery rate < 5 %.

RT-PCR

cDNA synthesis

RT-PCR was performed on the same RNA samples as used for Micro Array analysis. cDNA was synthesised using the SuperScript First Srand Synthesis System (Invitrogen). 1µg RNA was incubated in [10mM dNTP, 50ng random hexamers] for 5 minutes at 65°C, chilled on ice and add to [2µl 25nM $MgCl_2$, 4µl 0,1M DTT, 1µl RNAse inhibitor, 1µl MoMLV reverse transcriptase], incubated for 50 minutes at 42°C. RNA was purified using Qiaquick PCR Purification Kit (Qiagen) following the supplied protocol.

RT-PCR

RT-PCR was done with a LightCycler Sytem (Roche) using the Light Cycler Taqman Master Kit (Roche), flowing the supplied protocol.
following primers were used to detect the expression of respective genes:

β-actin	aaggccaaccgtgaaaagat tggtacgaccagaggcatac
Cdk6	tttcagatggcccttacctc acaggggtggcatagctg
Conductin	gcaggagcctcacccttc tgccagtttctttggctctt
CyclinD1	tctttccagagtcatcaagtgtg gactccagaagggcttcaatc
FGF15	ggcaagatatacgggctgat tccatttcctccctgaaggt
Gli1	tggaggtctgcgtggtaga ttgaacatggcgtctcagg
Hes1	tgccagctgatataatggagaa ccatgataggctttgatgacttt
Hes5	cagcccaactccaagctg agtcctggtgcaggctctt
Patched1	ggaaggggcaaagctacagt tccaccgtaaaggaggctta
R-spondin1	cgacatgaacaaatgcatca ctcctgacacttggtgcaga
TTR	catgaattcgcggatgtg gatggtgtagtggcgatgg

Primers for RT-PCR were obtained from Sigma

Results

Time and region specific functions of β-catenin in the developing cortex

Wnt signalling in the developing cerebral cortex

Of the 19 Wnt ligands discovered in mouse, at least 6 are expressed within the cerebral cortex during development. Two of these, Wnt2b and 3a, are solely expressed in the most medial part of the cortex, the cortical hem, throughout development (Fig.1a-f). Wnt8b is expressed in the hippocampal anlage and the cortical hem early during development (Fig.1n) and becomes progressively restricted till it is only expressed in the cortical hem at E15 (Fig.1n-p). In contrast to these Wnt ligands, Wnt7a and 7b are expressed throughout the cortex. While Wnt7a is expressed in the VZ precursors of the cortex from E13 on (Fig.1g-i), Wnt7b is expressed early in development (E11) in the medial VZ (Fig.1k) and becomes restricted to the neuronal layers of the cortex at later time-points (Fig.1l,m). Both ligands, Wnt7a and 7b, are additionally expressed in the VZ of the GE (Fig.1h-m). Some of the Wnt ligands, including Wnt8b, 2b, or 3a were reported to act predominantly through the canonical Wnt signalling pathway via β-catenin (Lee et al. 2000; Cho and Cepko 2006; Lee et al. 2006). Although β-catenin is ubiquitously expressed in the precursor cells of the developing cortex (Chenn and Walsh 2002), canonical Wnt signalling occurs in a descending gradient from caudo-medial to rostro-lateral, with the highest activity in the hippocampus anlage adjacent to the Wnt rich cortical hem. This can be observed by the gradient expression of Conductin, a direct downstream target of β-catenin (Fig.1r-t) (Lustig et al. 2002; Lustig and Behrens 2003), and by the activity of a reporter construct for canonical Wnt signalling (Fig.1u-w). The mouse line Tg[Fos-lacZ]34Efu (DasGupta and Fuchs 1999) carries the Topgal reporter construct that expresses β-galactosidase under the control of three TCF/LEF promoter elements and shows highest β-galactosidase expression and activity in the medial cortex, descending laterally (Fig.1u-w). The expression of both, Conductin and the Topgal reporter becomes increasingly restricted during development towards medial regions of the cortex (Fig.1r-w).

To investigate the role of canonical Wnt signalling during the development of the dorsal telencephalon I deleted β-catenin, the central player of the canonical Wnt signalling pathway, using a mouse line in which exons 2-6 of the β-catenin gene were flanked by loxP sites (B6.129-Ctnnb1tm2Kem/J, Brault et al. 2001). This mouse line was crossed to three different

mouse lines expressing the Cre-recombinase (referred to as Cre) gene at different stages and in different regions, to understand how β-catenin acts in a time and region specific manner. The Cre-lines used were the Emx1::Cre (Iwasato et al. 2000), the hGFAP::Cre (Zhuo et al. 2001) and the Nestin-Cre (Tronche et al. 1999) mouse line. In the Emx1::Cre line Cre is expressed from around E10 onwards and is restricted to the precursor cells of the developing cortex (Fig.2a) (Iwasato et al. 2000), consistent with the expression of Emx1 (Fig. 6c,d) (Yoshida et al. 1997). Cre expression, driven by the hGFAP promoter, starts around E12 at the medial cortex and expands throughout the lateral cortex till E13. At late neurogenesis (E16/E17) it becomes additionally expressed in the ventral telencephalon as well (Fig.2b) (Zhou et al. 2006). The two resulting conditional β-catenin mutants Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ were compared to specifically address the function of β-catenin at the onset (E11) and at the peak of neurogenesis (E14) in the developing cortex. In addition, they were compared to a third conditional β-catenin mutant, the Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant, to examine the role of canonical Wnt signalling within the cortical hem. In contrast to the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant, that delete β-catenin also within the cortical hem, Cre is not expressed in this region when driven by the Nestin enhancer (Tronche et al. 1999). Thus in the Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant β-catenin was deleted from the dorsal and ventral telencephalon but persisted in the cortical hem.

Deletion of β-catenin at the onset of neurogenesis and at midneurogenesis

The Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant, were used in a comparative approach to address the time-specific functions of β-catenin within the cortex, as they lose β-catenin at different developmental stages, at the onset of neurogenesis (E11) and at midneurogenesis (E15) respectively. Cre expression in the Emx1::Cre mouse line deleted β-catenin in the neocortex, the hippocampal anlage and the cortical hem but excluded the Choroid plexus (ChP). In these regions β-catenin protein was no longer detectable by immunostaining at E11 (Fig.2c,c'), consistent with the onset of Cre expression around E10 (Iwasato et al. 2000). In contrast, recombination by hGFAP-Cre deleted β-catenin protein from the cortex at E15 but included the ventral telencephalon at late embryonic stages (E16/E17) (Fig.2d,d'). In both mutants β-catenin was not detectable in the region of the cortical hem (arrows in Fig.2c',d').

To confirm that the deletion of β-catenin by both Cre lines indeed abolished canonical Wnt signalling, the expression of the canonical Wnt target gene Conductin and the activation of the Topgal reporter construct were analysed. The expression of Conductin, monitored by *insitu*

hybridisation, revealed expression in the WT cortex at E11 in a medial to lateral gradient but was completely absent from the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (Fig.2e,e'). This was confirmed by the lack of β-galactosidase in the E11 Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$/Topgal cortex (Fig.2g,g'). In the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex Conductin and β-galactosidase activity were both greatly reduced at E15 with small patches of Topgal activity maintained in the cortex (Fig.2f,f',h,h'). Conductin expression was absent from the cortex but some expression remained in the most medial tip of the cortical hem (Fig.2f,f'). Taken together, the deletion of β-catenin by Emx1::Cre and hGFAP-Cre resulted in a loss of canonical Wnt signalling at early and late stages of neurogenesis, respectively.

Early deletion of β-catenin within the entire developing cortex caused reduced tangential expansion of the neocortex

A gross analysis of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant telencephalon revealed that the medial to lateral expansion of the mutant cortex was already curtailed at E11 (Fig.3a,a'). At E13, the size of the mutant cortex was maximally decreased to 42% of that of WT littermates (Fig.3b,b'; Fig.4i) and hardly grew until the day of birth (P0) (Fig.3c-d'). Other brain regions, in contrast, developed normally as e.g. the ventral telencephalon and the diencephalon (Fig.3c-d'), where Emx1::Cre was not expressed and β-catenin was still present. The reduction in cortical size in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ was more moderate compared to that in Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants as the maximum reduction in size observed was 68% only (Fig.3e-f';Fig.4i). In both mutants the medial cortex with the hippocampal anlage was reduced in size, consistent with the important role of canonical Wnt signalling in the development of the hippocampus as known from Wnt3a and Lef1 loss of function analyses (Galceran et al. 2000; Lee et al. 2000) and deletion of β-catenin specifically in the hippocampus (Machon et al. 2003). The differential reduction in cortical expansion in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant showed that the extent of phenotypic malformation, caused by the deletion of β-catenin, was highly depended on the developmental stage at which β-catenin was deleted. This indicated that the function and importance of β-catenin mediated signalling changes during development, even within a concise region such as the cerebral cortex.

Stage dependent influence of β-catenin on precursor cell proliferation

In general, impaired growth of tissue and organs can be caused by reduced proliferation, premature differentiation and ectopic cell death. As canonical Wnt signalling has been

implicated in all three of these processes, proliferation, differentiation and apoptosis were analysed in the β-catenin deficient cortices, to reveal the cause of the reduced cortical expansion in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants. Proliferating cells and neurons were visualized by immunostaining for the proliferation marker Ki67 and the early neuronal marker β-III-tubulin, respectively (Fig.4a,b). While precursor cells in the WT cortex reside in the germinal layers separated from the neurons in the cortical plate this cortical architecture was disturbed in β-catenin deficient cortices (Fig.4a,a',b,b'). In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant a broad band of neurons in the centre of the cortex was surrounded by a band of precursor cells that intermingled with neurons at the ventricular surface (Fig.4a,a'). In the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex by contrast β-III-tubulin positive neurons were localized randomly within the germinal zones (Fig.4b,b'). Despite this, some remnants of the cortical plate were present in this mutant probably because they had been established prior to β-catenin deletion (Fig.4b,b', arrow in b'). *Insitu* hybridisation for Lhx2 and Hes5, expressed by cortical precursor cells, confirmed the disintegration of the cortical architecture, as Lhx2 expressing cells covered a broad area of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex, and Hes5 cells, although extremely reduced in number were scattered throughout the cortex (Fig.4e,e'). Similarly Hes5 positive precursor cells in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex were not restricted to the VZ but scattered at a larger distance from the ventricle compared to WT (Fig.4f,f').

These *Insitu* hybridisation analyses also confirmed the pronounced reduction of precursor cells, especially in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex, that had been already apparent from the Ki67 immunostaining. To quantify the proliferation of precursor cells in the β-catenin mutants, mitotic cells were labelled by an antibody binding to the phosphorylated form of histone 3 (PH3) which is specifically phosphorylated during M-Phase (Fig.4g,h). As precursor cells of the VZ and the SVZ undergo mitosis along the ventricular surface and at basal positions respectively, PH3 immunstaining revealed two bands of mitotic cells in the WT cortex (Fig.4,g,h). Deletion of β-catenin abrogated this pattern and mitotic cells were distributed irregularly within the cortex (Fig.4g',h') consistent with the immunostaining for Ki67 and the *insitu* hybridisation for Hes5 (Fig.4f'), indicating that the separation of VZ and SVZ was lost in the β-catenin deficient cortices. For the quantitative analysis, the number of PH3 positive cells was normalized to the size of the cortex, measured with the Neurolucida software 6.0. The number of PH3 positive cells in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex at E13 was reduced to 27.6% of WT levels in the caudal cortex (Fig.4i). Similar, although less pronounced was the reduction of PH3 positive cells in the caudal hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6\text{-}flfl}$ cortex at E15, where it was reduced to 67.7% of WT levels (Fig.4i). Interestingly, in both mutants

proliferation was most extensively reduced at caudal levels with a reduction of PH3 positve cells to 27.6% caudally compared to 51.4% rostrally in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$, and 67.7% caudally compared to 88.0% rostrally in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6\text{-}flfl}$ mutant cortex (Fig.4i). Consistently, cortical size in both mutants was stronger reduced caudally than rostrally. The rostral to caudal difference in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant might be correlated to a gradient expression of Cre, as Emx1 is expressed in higher levels caudally than rostrally (Bishop et al. 2002). Despite this possibility, the pronounced phenotype of β-catenin deletion was strikingly consistent with the high expression of Wnt ligands in the medio-caudal cortex and the development of the hippocampal anlage in this area, that was shown to be highly dependent on Wnt signalling (Galceran et al. 2000; Lee et al. 2000; Machon et al. 2003). Thus in agreement with the differently extensive reduction in cortical size in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6\text{-}flfl}$ mutants, precursor proliferation was affected more when β-catenin was lost early during cortical development, suggesting that cortical precursor cells were less sensitive to a reduction in canonical Wnt signalling at progressively later stages in corticogenesis.

β-catenin controls the cell cycle re-entry of cortical precursor cells

Canonical Wnt signalling has been suggested to control cell cycle re-entry because over-expression of a stabilized form of β-catenin was shown to cause cortical overgrowth due to reduced cell cycle exit (Chenn and Walsh 2002; Chenn and Walsh 2003). Therefore I examined whether the observed reduction in precursor cells in the β-catenin deficient cortices was caused by an increased cell cycle exit and premature neurogenesis. The Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant was chosen for this analysis as it showed the most dramatic effect on precursor proliferation. To reveal the immediate effect of the loss of β-catenin and to exclude possible secondary effects, mutant cortices were analysed at E11, shortly after the loss of β-catenin protein. At this stage, neurons, labelled by β-III-tubulin, were present in both WT and Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortices but were spread throughout the mutant cortex instead of being localized towards the basal side (Fig.5a,a'). In addition neurons were almost doubled in number in the β-catenin deficient cortex (Fig.5c). To determine the extent of cell cycle exit, the DNA-base analogue 5'-bromo-2-deoxyuridine (BrdU) was administered at E10 and the mice were examined 24h later. Immunostaining for BrdU and the neuronal marker β-III-tubulin was used to determine the proportion of precursor cells that had been cycling and thus incorporated BrdU, but left the cell cycle within the last 24h and adopted a neuronal identity (Fig.5b,b'). In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex twice as many cells had left the cell cycle

between E10 and E11 compared to WT cortex (Fig.5c), indicating that the deletion of β-catenin induced cortical precursor cells to prematurely exit the cell cycle and differentiate into neurons. Thus, in the absence of β-catenin the cortical precursor pool was depleted, leading to the extensively reduced number of precursor cells observed at E13. These results are consistent with previous observations of gain and loss of function analyses of β-catenin (Brault et al. 2001; Chenn and Walsh 2002; Zechner et al. 2003) although one study reported unchanged levels of cell cycle exit at late developmental stages, upon the loss of β-catenin (Machon et al. 2003).

Loss of β-catenin increased cell death during early cortical development

In addition to controlling proliferation and cell cycle exit, β-catenin has been reported to cause cell death when lost during early development due to its role in cellular adhesion (Junghans et al. 2005). To reveal whether cell death contributed to the extensive reduction in cortical size observed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant cortex, the number of Tunel- or activated Caspase3-positive cells was quantified at E11. This showed that cell death was more than doubled upon deletion of β-catenin by Emx1::Cre compared to WT (Fig.5c). Taken together, this data demonstrates that the reduced cortical size, observed after deletion of β-catenin at the onset of neurogenesis is caused by increased apoptosis as well as premature cell cycle exit and differentiation of cortical precursor cells. Thus, β-catenin exerts a concerted effect on all three key processes of development.

Loss of β-catenin after the onset of neurogenesis did not affect dorso-ventral patterning

In addition to proliferation, differentiation and cell death, canonical Wnt signalling was reported to participate in dorso-ventral patterning of the telencephalon (Backman et al. 2005). Deletion of β-catenin prior to neurogenesis (at E8) within the telencephalon was reported to induce ventralisation of the cortex. This was not the case when β-catenin was deleted later than E11. As deletion of β-catenin by Emx1::Cre started around E10 patterning defects may have contributed to the phenotype observed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. To investigate this, mRNA and protein of genes specifically expressed in the dorsal or ventral telencephalon were examined. As previously described Pax6, Emx1, 2 and Ngn2 were expressed in the dorsal but not in the ventral telencephalon of WT embryos at E13 as well as at E15 (Fig.6.a-h) (Walther and Gruss 1991; Simeone et al. 1992; Stoykova and Gruss 1994; Mallamaci et al.

1998; Bishop et al. 2000; Fode et al. 2000). Although expression of these genes was reduced in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant cortex, due to the reduction in the total number of precursor cells, the remaining precursors expressed Pax6 and Ngn2 (Fig.6a',g'), and also Emx1 and 2, albeit at lower levels (Fig.6c',e'), demonstrating that β-catenin deficient cortical precursor cells maintained their dorsal identity. The relatively pronounced reduction of Emx2 in comparison to Pax6 and Ngn2 might be explained by the direct regulation of Emx 2 by canonical Wnt signalling (Theil et al. 2002; Muzio et al. 2005). Consistent with the maintenance of a dorsal identity, precursor cells in the Emx1$^{Cre\ /\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex did not express ventral TF such as Mash1, that remained restricted to the ventral telencephalon in mutant as in WT (Fig.6l,l'). Thus, precursor cells in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex were specified normally, demonstrating that dorso-ventral patterning of the telencephalon is not dependent on canonical Wnt signalling after the stage of E10. Consistent with this, cortical precursor cells in the hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6\text{-}fl/fl}$ mutant cortex maintained the expression of Pax6, Emx1, 2 and Ngn2 and did not express Mash1 (Fig.6b',d',f',h'), although they displayed a relatively stronger decrease in the expression of Emx1 and 2, similar to the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant.

These data demonstrate that dorso-ventral patterning of the telencephalon was not affected in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant, suggesting that canonical Wnt signalling does not interfere with dorsal-ventral patterning once it has been established.

Deletion of β-catenin by Nestin-Cre, excluding the cortical hem

The deletion of β-catenin using the hGFAP-Cre and the Emx1::Cre lines allowed me to address the function of β-catenin during progressing neurogenesis but within the same telecephalic regions, namely the entire cerebral cortex. This included the deletion of β-catenin from the most medial edge of the cortex, the cortical hem, that expresses several Wnt ligands (Fig.1) and has been suggested to function as a cortical signalling centre (Grove et al. 1998). The cortical hem itself furthermore expressed the canonical Wnt signalling target Conductin at E12 as well as E15 but did not show Topgal reporter activity (Fig.7a-d). In a second Wnt reporter mouse line (Maretto et al. 2003) however, the cortical hem displayed reporter activity at E11.5 but not at E16.5 (Machon et al. 2007), suggesting that these differences may be due to the variability in reporter activity.

To examine whether β-catenin serves additional functions in this signalling centre, I investigated whether excluding Cre-recombination from the cortical hem would change aspects of the phenotypes observed upon deletion of β-catenin by other Cre lines. Cre

recombination by the Nestin-Cre line deleted β-catenin in the ventral telencephalon from E12 and in the cortex from E13 onwards but excluded the most medial part of the cortex, the cortical hem (Fig.8a,a',b,b'). Consistent with the loss of β-catenin in the neocortex and the hippocampus anlage, Topgal reporter activity was strongly reduced in the E13 Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (Fig.8c,c'). Similarly Conductin expression was lost from the hippocampus anlage but persisted in the cortical hem at E15, as did β-catenin (Fig.9 a,a',b,b'). Comparisons of Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants to Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants revealed that the morphology of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex was strikingly different from the other two (Fig.9c,c'; compare to Fig.3). The Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex was not reduced in its tangential expansion but appeared thinner in lateral and caudal regions. Despite this, the cortical size, measured in μm^2 on telencephalic sections was solely reduced at the most caudal end of the cortex (Fig.9d) and the quantification of the number of PH3 positive cells revealed a slight reduction only in the medial regions of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (Fig.9d). Thus despite the loss of β-catenin in the developing cortex at E13/E14, comparable to that in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant, the effect on cell proliferation and the lateral extension of the cortex was less pronounced. However the cortical architecture of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant was disrupted and resembled the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. Similar to the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6f/lfl}$ cortex, the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex displayed a thin cortical plate beneath which neurons and precursor cells were found to intermingle (Fig.10a,a'). Additionally, *insitu* hybridisation for Lhx2 and Hes5 as well as immunostaining for PH3 confirmed the similarity in cortical architecture between the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. Furthermore, also the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant did not show any defects in dorso-ventral patterning consistent with the late deletion of β-catenin (Fig.11a-d'). Pax6 and Ngn2 were both expressed by cortical precursor cells (Fig.11a,a',d,d') whereas Emx1 and 2 were reduced in their expression levels, similar to the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6f/lfl}$ mutant (Fig.11b,b',c,c').

Besides the similarities in cortical architecture and the differences in cortical morphology, the relative minor effect on precursor cell proliferation in the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex compared to the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant suggested that canonical Wnt signalling within the cortical hem may promote proliferation troughout the developing cortex. In this regard it was of particular interest whether the cortical hem itself was disrupted in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex but maintained in the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. Therefore the expression of Wnt ligands present in the medial cortex was examined by *insitu* hybridisation, to reveal whether the cortical hem was lost in

any of the described mutants. In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex, that displayed the strongest abnormalities in cortical morphology, Wnt2b expressing cells were observed, located immediately adjacent to the Choroid plexus (Fig.12a'). This resembled the localisation of Wnt2b in the cortical hem in WT, where it is localized between the hippocampus anlage laterally and the Choroid plexus medially (Fig.12a,b,c). Similar, Wnt2b expression was detected in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex as well. The presence of cortical hem cells in all three mutants was confirmed by the expression of Lmx1a (Fig.12d-f'). In contrast to the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex however, the expression of a second Wnt ligand expressed in the cortical hem, Wnt3a, was not detectable in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex, suggesting that the early deletion of β-catenin affected the cortical hem more extensively than the later deletion by hGFAP-Cre. Two further genes were investigated that are not restricted to the cortical hem but also expressed in the medial cortex, Wnt8b and Id3 (Fig.12k-p). In all three mutants the expression of both genes was greatly reduced, but persisted in the region where Wnt2b and Lmx1a were expressed as well (Fig.12k'-p'), arguing that the lack of canonical Wnt signalling caused the loss of the hippocampal anlage. Thus the expression of Wnt8b and Id3 was only detected in the cortical hem of the β-catenin mutants. Besides Wnt ligands, the cortical hem also expresses other signalling molecules, for example BMP proteins. Interestingly, the expression of BMP6 in the cortical hem was lost in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant (Fig.12q,q',r,r') but persisted in the cortical hem of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (Fig.12s,s'). Thus, despite the persistence of cortical hem cells in the absence of canonical Wnt signalling, deletion of β-catenin in the cortical hem itself interfered with the expression of signalling molecules from this tissue and might therefore contribute to the strong cortical phenotype observed in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant.

The loss of β-catenin did not induce a change in progenitor identity

In agreement with previous studies (Backman et al. 2005), the experiments presented here showed that deletion of β-catenin at stages later than E10 did not affect dorso-ventral patterning of the telencephalon. However with the onset of neurogenesis NE cells become replaced by RG cells, which are capable of giving rise to neurons directly or via the generation of IP cells. As β-catenin deficient precursor cells prematurely leave the cell cycle, this could occur by generating two neurons directly, omitting the generation of IP cells. Alternatively, Wnt signalling could promote RG cell fate versus that of IP cells, as it has been suggested lately (Wrobel et al. 2007), causing a premature generation of IP cells at the

expense of RG cells. To address this question, RG cells were identified by immunostaining for the TF Pax6 (Akazawa et al. 1992; Götz et al. 1998; Englund et al. 2005), and IP cells by their expression of Tbr2, Cux2, NeuroD or Svet1 (Tarabykin et al. 2001; Nieto et al. 2004; Schuurmans et al. 2004; Englund et al. 2005). As described above, cortical precursor cells were correctly specified in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$, the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant cortex as they expressed the specific dorsal TF Pax6 and Ngn2 (Fig.6a-b',g-h';Fig.11a,a',d-d'). *Insitu* hybridisation for Svet1 (Fig.13a-c'), Cux2 (Fig.13d-f'), NeuroD (Fig.13g-i') and immunostaining for Tbr2 (Fig.13k-m') revealed that IP cells were specified in all three mutants, suggesting that both, RG cells and IP cells were generated in the absence of canonical Wnt signalling. However, as β-catenin deficient precursor cells were scattered throughout the mutant cortex it could not be excluded that they acquired a mixed identity, expressing genes that were normally confined to either RG or IP cells. To clarify this, double staining for Pax6 and Tbr2 was used to quantify the proportion of RG and IP cells (Fig.13n,n'). This analysis was done in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex, as it showed the most pronounced phenotype. In the E13 WT cortex about 67% of all cortical progenitors expressed Pax6 and thus had a radial glial identity, whereas about 27% were IP cells, expressing Tbr2 (Fig.13o). A small proportion of cells, about 7%, expressed both genes, probably undergoing transition from a RG to an IP cell fate. Most strikingly, the proportion of each population did not change upon the deletion of β-catenin (Fig.13o'), demonstrating that the extensive cell cycle exit induced at E11 did not prevent the generation of IP cells. Moreover this strongly suggests that canonical Wnt signalling does neither actively prevent nor induce RG cell fate, but rather favours cell cycle re-entry.

Neuronal subtypes in the β-catenin deficient cortex

The presence of RG and IP cells raised the question, whether specific neuronal subtypes could be generated in these mutant cortices. During neurogenesis different neuronal subtypes are generated at distinct developmental stages and settle in specific cortical layers with respect to their identity. The cortex of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant lacked this laminated structure and was considerably smaller at P0 compared to WT. To examine whether neurons of different subtypes were generated, neuronal subtypes were identified by the expression of specific genes. In P0 WT cortices, neurons of Layer I, positive for Reelin and Calretinin, were located directly beneath the pial surface (Fig.14a,b). In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex Reelin and Calretinin positive neurons were present and mostly located in proximity to the pial surface, although some were located within the cortical parenchyma (Fig.14a',b'). Neurons generated

later such as those of layers V and VI, were identified by the expression of ER81 and Tbr1 respectively (Fig.14e,f). Interestingly, despite the high numbers of precursor cells exiting the cell cycle early during development, not all neurons in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex expressed Er81 or Tbr1 (Fig.14e',f'). Moreover a high number of neurons expressed Cux2 or Satb2, genes normally restricted to neurons destined for upper layers of the cortex (Fig.14c,c',d,d'). These findings demonstrate that neurons of diverse neuronal subtypes can be generated from β-catenin deficient precursor cells, indicating that Wnt signalling is not biasing neurogenesis to one specific neuronal subtype. However upper layer neurons are mostly generated during mid- and late neurogenesis when the number of precursor cells in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex was already strongly reduced. This suggested that Cux2 and Satb2 expressing cells in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6\ fl/fl}$ cortex were either generated by precursor cells that persisted despite the lack of Wnt signalling or they were generated early in neurogenesis but acquired an upper layer neuronal fate. To determine the time point when these cells were generated BrdU was administered to pregnant mice at E11 or E16, thereby labelling cells exiting the cell cycle at the respective developmental stage (Fig.15a-f'). As expected from the high rate of cell cycle exit between E10 and E11 many neurons in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex were labelled by BrdU at E11 with many of them expressing Tbr1 (Fig.15c,c'). In contrast however to neurons generated at E11 in the WT cortex, some of these neurons in the β-catenin deficient cortex expressed Cux2 (Fig.15a,a'). This gene is normally restricted to upper layer neurons that are mostly generated from E14 onwards. Consistently, neurons generated in the WT cortex at E16, expressed Cux2 although they had not yet reached the basal surface of the developing cortex to establish layer II/III (Fig.15d). Similarly, many neurons generated at E16 in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex expressed Cux2 (Fig.15d') but also Er81 and Tbr1 (Fig.15e',f'), suggesting that the premature neurogenesis occurring upon the loss of Wnt signalling impairs the mechanisms controlling neuronal subtype specification. Besides these alterations, it should be noted that a remarkably high number of neurons were generated at the end of neurogenesis in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex (Fig.15d'-f'). This demonstrates that the increased cell cycle exit at early developmental stages did not entirely deplete the pool of cortical precursor cells. Moreover, GFAP positive cells were present in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant cortex at P0 suggesting that precursor cells were maintained in the mutant cortex throughout neurogenesis and generated astrocytes afterwards (Fig.14 g,g'). Taken together, these data suggest that a small subset of cortical precursor cells did not immediately exited from the cell cycle upon the loss of β-catenin but persisted throughout neurogensis, generating upper layer neurons and astrocytes. Moreover different cortical

neuronal subtypes were generated in the absence of canonical Wnt signalling although with certain anomalies in the correlation of neuronal specificity and generation time point.

The data presented here are generally consistent with observations from the development of various tissues as well as from cancer (Huelsken and Birchmeier 2001; Pires-daSilva and Sommer 2003; Reya and Clevers 2005; Brembeck et al. 2006; Clevers 2006) consolidating the function of β-catenin mediated Wnt signalling in cell proliferation and cell cycle re-entry. Beyond that, they reveal that the functions of β-catenin during neurogenesis are time and also region specific and suggest a distinct role for the cortical hem. Independently however of the strength of the proliferative defects observed in the different conditional mutants, deletion of β-catenin always disrupted cortical tissue architecture. This was apparent from the scattered distribution of neurons and precursor cells and the absence of cortical lamination.

α- and β-catenin in adherens junctions formation and cell polarity

Besides the prominent function of β-catenin at the centre of canonical Wnt signalling, β-catenin is a major participant of AJs that are formed between NE and RG cells by the extra-cellular domains of Cadherin molecules. Intracellularly, Cadherins bind to β-catenin which in turn is connected to the cytoskeleton by α-catenin (Drees et al. 2005; Yamada et al. 2005; Weis and Nelson 2006). Thus, β-catenin serves fundamental functions in the regulation of canonical Wnt signalling as well as cell adhesion and motility. However these multiple functions make it difficult to allocate the various malformations observed in the β-catenin mutants to one specific molecular function of β-catenin. As the disintegration of AJs was previously shown to induce changes in cell morphology and disruption of epithelial structures (Kadowaki et al. 2007; Takeichi 2007) I assumed that the disorganisation of the developing cortex upon deletion of β-catenin was due to its function in cell adhesion. To examine this, I decided to compare the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6\ fl/fl}$ mutant to a second conditional mutant lacking a molecule that shares the function of β-catenin in AJs but not in canonical Wnt signalling. Therefore I used the Emx1::Cre line to delete α-catenin in the developing cortex and compared the two conditional mutants: $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$.

Loss of α- or β-catenin impaired cortical laminar architecture similarly

Recombination by Emx1::Cre eliminated the α- and β-catenin protein from the cortex of $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants respectively (Fig.16a-d'). The early

deletion of β-catenin by Emx1::Cre caused the disturbance of cortical architecture, such that neurons and precursor cells intermingled instead of sorting to the neuronal and germinal layers respectively. This was already apparent at E11 when neurons started to accumulate at positions close to the ventricular surface (Fig.5a,a') and became even more pronounced at E13 (Fig.4a,a'). Similar defects were found in the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant with a comparable progression of defects in cortical organisation as in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant (compare Fig.3 and Fig.4 to Fig.23). In addition, germinal layers lost their internal organisation, as apparent from the irregular distribution of PH3 positive cells and the immunostaining for Pax6 and Tbr2 in the α- and β-catenin mutant cortices (compare Fig.3.&4 to Fig.23). This demonstrates, that VZ and SVZ progenitors were not spatially separated but localized throughout the mutant cortices (Fig.13n,n'; Fig.28d,d'). Thus, α- and β-catenin were both essential to cortical organisation and their deletion affected cortical lamination similarly.

Maintenance of cell-cell contacts despite the lack of α- or β-catenin

As both molecules interact to form AJs at the apico-lateral membrane of NE and RG cells and epithelial cells have been reported to rely on AJs to maintain their structures, the state of AJs in the catenin mutant cortices was examined by light and electron microscopy. The electron microscopic analyses were performed in collaboration with Dr. Michaela Wilsch-Bräuniger at the MPI for Molecular Cell Biology, Dresden and Dr. Shakibaei at the Faculty of medicine at the LMU Munich.

Cadherins, the major components of AJs in the cortex (Redies and Takeichi 1996; Takeichi 2007), were detected with a pan-Cadherin antibody and Rhodamine labelled Phalloidin was used to visualize the F-actin filaments that are connected to the AJs (Chenn et al. 1998). Both molecules were localized at the apical side of WT NE and RG cells, delineating the ventricular surface at E11 and E13 (Fig.17a,b, Fig.18a-d) and Cadherins co-localized with α- and β-catenin consistent with the interaction of the three molecules to form AJs (Fig.17c,d). In the catenin deficient cortices however, Cadherins and F-actin were distributed irregularly within the tissue at E11 and E13, similar to α- or β-catenin in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex respectively (Fig.17a'-d', Fig.18a'-d'). Interestingly, Cadherins remained co-localized with β-catenin between neighbouring $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortical cells (Fig.18d'), indicating that both molecules were still able to form a complex and might thus form AJs, despite the loss of α-catenin. In the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortical cells in contrast, the co-localisation of α-catenin and Cadherins was not prominent, which is expected, considering that β-catenin links these molecules to each other.

To definitively examine whether AJs persisted in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex, mutant brains were examined by electron microscopy and compared to the cortex of WT animals. AJs in the WT cortex can be observed as elongated, electron dense structures along the apico-lateral membranes of neighbouring cells close to the ventricular surface (Fig.17e,;Fig.18e,f). Intriguingly, these structures were present between α-catenin-deficient cells, consistent with the localization of β-catenin and Cadherin observed by light microscopy (Fig.17e'). However in contrast to WT, cell-cell contacts in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ mutant cortex were distributed irregularly and most of them were localized within the cortical parenchyma (Fig.17e'). These cell-cell junctions could be observed at E11 as well as E13 arguing that the persistence of cell-cell contacts in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex was not due to remnants of α-catenin protein (Fig.17e';Fig.18f'). Thus, α-catenin is not essential for the formation and maintenance of AJs in RG cells. Indeed α-catenin was recently shown to bind β-catenin and F-actin not simultaneously but to shuttle between both molecules (Drees et al. 2005; Yamada et al. 2005). Therefore it is conceivable that cell-cell contacts can form and persist without α-catenin connecting them dynamically to the cytoskeleton. Nevertheless the highly similar phenotype in regard to cortical disorganisation observed upon the deletion of α- or β-catenin raised the question whether AJs could remain in the absence of β-catenin as well. Indeed electron microscopy demonstrated that cell-cell contacts, reminiscent of AJs, were present in the E13 Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex (Fig.18e,e'). These data suggest that Cadherins are sufficient to create cell-cell contacts between cortical cells in the absence of α- or β-catenin. However the loss of these molecules in the entire tissue weakened the coherence of cells such that they could not provide a stable epithelial scaffold.

Structural alteration of radial glial cells in catenin deficient cortices

The electron microscopic examination also revealed a prominent change in the morphology of the cells in both mutant cortices. Whereas in the WT cortex, RG cells exhibited a longitudinal bipolar shape (Fig.19a,b), cells in the α- and β-catenin mutant cortex were rather round shaped (Fig.19a',b'). This was consistent with the observation that cortical cells in both mutant cortices had round shaped nuclei in contrast to the elongated nuclei of WT cells (arrows in Fig.19a',b'). Furthermore immunostaining for the intermediate filament nestin showed that radial processes in both, the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex were reduced and disorganised (Fig.19c-d'). Notably, nestin immunoreactivity appeared to be generally reduced in both mutant cortices although the number of proliferating cells was decreased in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex only. Taken together with the impaired cortical

organisation of the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex these results suggested that RG cells lost their radial morphology in the absence of α- or β-catenin. To visualize the morphology of individual RG cells, DiI was used to label single cortical cells displaying the radial bipolar shape of WT cortical cells (Fig.19e,e). These experiments were done together with Dr. Leanne Godhino.

In contrast to WT RG cells, α-catenin deficient cells were round in shape with none or a few short processes (Fig.19e') and β-catenin deficient cells lacked longitudinal, bipolar processes but exhibit multiple small processes reaching out in diverse directions instead (Fig.19f'). Taken together, this suggests that RG cells, despite their ability to form AJs, cannot maintain their apical and basal process in the absence of α- or β-catenin, lacking the connection from the cell-cell contacts to the cytoskeleton. This may well contribute to disturbed cortical organisation observed in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ or Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex, as radial processes provide the scaffold new born neurons use to migrate out of the VZ.

Cortical cells lacking α- or β-catenin maintained certain aspects of cellular polarity

In addition to epithelial stability, AJs also serve as an anchoring point for multiple other molecules, many of which have been shown to function in cellular polarity such as Par proteins or Numb (Chenn et al. 1998; Macara 2004; Perez-Moreno and Fuchs 2006). The redistribution of AJs in the α- or β-catenin deficient precursor cells may therefore influence these molecules and thus disturb cellular polarity. To investigate this, I examined the localisation of Par3, which attachs the Par complex to the membrane. In the WT cortex Par3 is localized in proximity to Cadherins at the apical side of RG cells at E11 as well as at E13 (Fig.20a-d). In the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and the Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex Par3 was still present however not exclusively localized at the ventricular surface (Fig.20a'-d'). Nevertheless Par3 was distributed in patches and remained localized adjacent to Cadherins as observed in the WT cortex. Moreover Western Blot analysis revealed that the levels of Par3, aPKC and Par6, which form the Par complex together with Cdc42 (Manabe et al. 2002; Takekuni et al. 2003), were not changed in the Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex, neither was the protein amount of the phosphorylated form of aPKCλ/ι (Fig.21). As aPKC gets phosphorylated in an active Par complex (Wu et al. 2006) these data strongly suggest that a functional Par complex can be localised adjacent to AJs in the absence of α-catenin. These Western Blot analyses were done together with Franziska Weinandy.

Prominin (CD133), located to the apical surface of RG cells, delineates the ventricular surface in WT (Weigmann et al. 1997) (Fig.20e-h) and was present in both α- and β-catenin mutant

cortices, although it did not localize towards the ventricular lumen (Fig.20e'-h'). Similar to Par3, Prominin was localized unidirectional within the mutant cells indicating that it could be still positioned in an asymmetric manner. Thus RG cells, although lacking α- or β-catenin still possessed AJs together with a non-homogeneous distribution of apical membrane components, suggesting that these cells maintained at least aspects of cellular polarity, despite their impaired neuroepithelial organisation and radial morphology.

α-catenin deficiency alters proliferation but not neurogenesis of radial glial cells

The depletion of α-catenin and β-catenin from cortical precursor cells caused comparable aberrations in radial glial morphology. Despite the similar cortical architecture of the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant, both differed considerably in the size of the cortex. Whereas the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex was distinctly smaller, the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex was increased in size compared to WT at E11 (Fig.22a,a'). It was shorter in its tangential expansion but broadened in the radial axis. Interestingly the increase in cortical size was more prominent at rostral than at caudal levels (compare Fig.22a'-rostral to 16c'-caudal) Two days later, at E13, the cortical tissue of the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex had increased to three times the size of the WT cortex, most prominently anteriorly (Fig.22b,b'; Fig.23e). On the day of birth the mutant cortex was distinctly larger compared to that of WT littermates and at most rostral levels, the profoundly increased cortical tissue displaced the ventricular lumen (Fig.22c,c'). Thus deletion of α-catenin by Emx1::Cre caused the formation of an overgrown cortex, displaying a tissue-architecture similar to that of the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex.

α-catenin depletion did not induce the ectopic activation of entire cell signalling pathways

The loss of α-catenin has already previously been reported to cause ectopic tissue expansion due to hyperproliferation (Vasioukhin et al. 2001; Lien et al. 2006a). However, the mechanisms by which the loss of α-catenin had been suggested to causes hyperprliferation in skin and nervous tissue were not the same. Whereas the loss of α-catenin in skin was reported to lead to the activation of MAP-kinase signalling (Vasioukhin et al. 2001), in brain it has been claimed to induce the expression of SHH signalling targets in the cortex, resulting from the loss of AJs (Lien et al. 2006a). Moreover it has not been possible to assign a particular function to α-catenin in the SHH pathway nor in other signalling pathways, although it

interacts with strikingly many and different molecular partners (Perez-Moreno and Fuchs 2006). This suggests, that α-catenin serves very different functions by interacting with distinct molecules in a highly cell type specific manner. In this regard it should be noted that the α-catenin mutant previously reported (Lien et al. 2006a) lacked α-catenin throughout the entire CNS as the α-catenin gene was deleted by Nestin-Cre, affecting many different cell types. In contrast to this, I was able to investigate the distinct function of α-catenin in cortical precursor cells as the deletion by Emx1::Cre did not affect any other compartment of the CNS.

To investigate whether the loss of α-catenin exclusively in the cortex activated SHH or any other cell signalling pathway, gene expression in WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices was analysed by *insitu* hybridisation, microarray analysis and real-time-PCR (RT-PCR). mRNAs of FGF15, Gli1 and Patched1, genes regulated by SHH-mediated signalling (Gimeno et al. 2003; Marcucio et al. 2005; Fuccillo et al. 2006), were strongly expressed in the ventral GE, but were not detected in the cortex of WT or $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mice at E11 (Fig.24a-c'). Similarly, I did not observe quantitative differences in the mRNA levels of these genes in $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and WT cortices at E11 and E13 (Fig.24 d,e) and expression levels were 2-14 fold lower in the cortex compared to the GE of both $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and WT (Fig.24 d,e). Thus, deletion of α-catenin exclusively in the cortex did not activate this pathway, in contrast to deletion throughout the CNS including the GE (Lien et al. 2006a), suggesting that the latter is a non-cell autonomous effect. Next, FGF-mediated MAP-kinase signalling was examined, as previous deletion of α-catenin in the skin had activated this pathway (Vasioukhin et al. 2001) and the more prominent phenotype in rostral parts of the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex may be consistent with a role for FGF8 which is expressed highest at the rostral pole of the telencephalon (Fukuchi-Shimogori and Grove 2001). However, *insitu* hybridisation for the FGF downstream targets Pea, Sef and Erm (Tsang and Dawid 2004) revealed comparable expression in WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices at E11 (Fig.25a-c'). Moreover, alterations from immunolabeling for phosphorylated MAP-kinase were not apparent (Fig.25d,d'), suggesting that this pathway was not affected in the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. Similar data were obtained for the Notch signalling pathways, as Hes1 and Hes5 were still expressed at normal levels in the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex (Fig.26a,e,e'). To investigate global gene expression in WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices beyond the so far investigated signalling pathways, mRNA from E11 and E13 WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices, derived from three litters, was hybridized to the Affymetrix MOE430 2.0 arrays and data analyzed as described in the Methods. Surprisingly, only 34 genes were detected to be significantly differentially expressed between WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices at E11 (p-value ≥0,001)

with 68% up-regulated in the Emx1Cre/α-catΔex2fl/fl compared to WT cortex (Table a). The reliability of this analysis was confirmed by RT-PCR showing differential expression comparable to those observed in the micro array (three representative examples are shown in Fig.27). Interestingly this analysis did not reveal the activation of a distinct cell signalling pathway upon the loss of α-catenin, when restricted to the dorsal telencephalon. Although almost 50% of the genes differentially expressed in WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex were of unknown function, some genes were interesting. Among the genes that had been down-regulated in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex some are known to have functions in the nervous system such as FGF17, Transthyretin, R-spondin1, AldolaseC and Calveolin1, however they did not pinpoint to a particular pathway or neurogenic function. In contrast, the expression of two kinesin family member (Kif) genes, functioning in molecular transport towards the membrane and along axons (Hirokawa 2006), was up-regulated, with Kif5c reported to be neuron specific (Kanai et al. 2000). Moreover CyclinD1 and Musashi homolog2 (Sakakibara et al. 2001), both expressed in neural precursor cells as well as Jarid1a (jumonji) (Takahashi et al. 2007) that counteracts the pro-proliferative activity of CyclinD1 were also up-regulated in the mutant cortex. CyclinD1, was of particular interest as it is known to promote transition from G1 to S-phase and thus promotes proliferation (Gladden and Diehl 2005). It was expressed at a twofold higher level in the E11 Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex compared to WT (Table a; Fig.27). Interestingly this increase was no longer observed at E13, when only 5 genes were found to be differentially regulated with no overlap with the 34 differentially regulated genes at E11 (Table b). Moreover 4 of these genes are located on the two sex chromosomes, indicating that the most pronounced difference between WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant at E13 was the sex. However the sex of the analysed embryos had not been determined in advance.

Thus, the data from the micro array suggested that the deletion of α-catenin promotes proliferation by an increase in CyclinD1 expression but did not indicate the activation of a specific extrinsic signalling pathway by which CyclinD1 could be controlled.

However, α-catenin closely interacts with β-catenin, that in turn is conducting canonical Wnt signalling, and CyclinD1 is a prominent target of this signalling pathway. Therefore it was possible that during the rearrangements of AJs upon the deletion of α-catenin, β-catenin was released from the membrane and freed to translocate into the nucleus. To investigate whether β-catenin gene transcription was activated in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant, this was crossed to the Topgal reporter mouse line. Interestingly this revealed that Topgal reporter activity was comparably strong in Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and WT at caudal levels of the cortex, whereas

activity was considerably increased in the rostral Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex. In this regard it should be noted that the increase in cortical size was most prominent at rostral levels in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex (Fig.23e). Despite this ectopic reporter activation in the rostral cortex of the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ mutant other Wnt signalling targets but CyclinD1 were not differentially expressed between WT and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex nor was Conductin expression found to be increased (Fig. 26d'). Taken together it appears conceivable that the increased expression of CyclinD1 in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex is enhanced but not exclusively caused by a β-catenin dependent mechanisms.

Loss of α-catenin transiently accelerated the cell cycle

The increased cortical size together with the increased expression of CyclinD1 prompted me to examine proliferation in the E11 Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex. The number of mitotic cells per area was significantly increased in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex compared to WT at E11 (Fig.23e.). Given the increase in CyclinD1 expression and the absence of any difference in cell death (Fig.23e) a shorter cell cycle length might have caused the increase in proliferation. To examine this, cells in S-phase were monitored by the application of BrdU 30 minutes prior to sacrifice. The proportion of BrdU positive cells amongst all proliferating cells was calculated as the labelling index (LI), an indicator of the length of the cell cycle. Notably, the LI is almost doubled in E11 Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ compared to WT cortex (Fig.23e) suggesting that Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ precursor cells have a shorter G1 phase. Moreover deceleration of the cell cycle has been correlated a neurogenic precursor fate (Calegari et al. 2005; Lukaszewicz et al. 2005) suggesting that a reduced number of neurons were generated in the α-catenin deficient cortex. Indeed, the number of β-III-tubulin positive neurons in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex at E11 was decreased by 40% compared to the cortex of WT littermates (Fig.23e). Interestingly, by E13 the ratio of neurons to precursor cells in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex was similar to that of WT as the number of PH3 positive cells per area was similar between WT and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortices (Fig.23e). This is consistent with the return of CyclinD1 to normal expression levels in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex at E13. Thus, the loss of α-catenin led to an immediate but transient acceleration of the cell cycle, increasing the pool of progenitors and the size of the cortex.

Progenitor and neuron fate in the absence of α-catenin

In contrast to the deletion of β-catenin, the loss of α-catenin caused a transient increase in precursor proliferation. However, the increased cell cycle exit in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$

mutant cortex still allowed the generation of IP cells and did not alter the ratio of Pax6 to Tbr2 positive precursor cells (Fig.13n,n',o,o').Thus I analysed whether IP cells were still generated if RG cells cycle faster. Progenitor fate was examined in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex as it was done in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. Similar to the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex RG and IP cells were present but scattered in Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex (Fig.28.a-d'). Consistently with the data obtained from E13 WT littermates of Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants the majority of progenitors in WT litter mates of Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutants, were Pax6-positive and only 20% of all progenitors were Tbr2-positive with little overlap (Fig.28e) and again the composition of the progenitor pool in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex was strikingly similar to that of WT (Fig.28e'). An apparent aberration in this marker analysis however, was the increase in the proportion of cells expressing both, Pax6 and Tbr2 at the expense of Tbr2 only expressing cells. Thus, deletion of α-catenin allowed the generation of IP cells but prompted the continuous expression of Pax6. Despite this, the presence of Pax6 and Tbr2 positive precursor cells indicated that α-catenin, similar to β-catenin deficient RG cells maintained their fate and generated IP cells. IP cells in turn are believed to predominantly give rise to upper layer neurons late in neurogenesis whereas deep layer neurons are generated early during neurogenesis, supposedly directly from RG cells (Nieto et al. 2004; Schuurmans et al. 2004; Wu et al. 2005). Taken the reduced neurogenesis in early development and the slight changes in IP cell gene expression together, it was of interest whether the respective neuronal subtypes were generated in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. Neurons populating layer I, expressing Reelin and/or Calretinin were situated beneath the pial surface in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex, similar to WT (Fig.29a-b'). Neurons generated later such as those of layers V and VI, identified by the expression of ER81 and Tbr1 respectively (Fig.29c,d,e), appeared in clusters throughout the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex, sometimes even at superficial positions near the pial surface (Fig.29c',c'',d',d'',e',e''). Neurons of the upper cortical layers II and III, Cux2 and Satb2 positive were present but scattered in the P0 Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex, with some cells localized at superficial positions (Fig.29f',f'',g',g''). Thus, different subtypes of cortical neurons, although miss-localized, were still specified in the absence of α-catenin. Furthermore, BrdU-birthdating analyses revealed that neurons born at E12 or E14 were scattered or clustered, consistent with their generation after the deletion of α-catenin and the consequent disorganisation of RG cells (Fig.29d',d'',f',f'',g',g''). Despite this, BrdU labelled cells still expressed their appropriate layer markers, as Cux2 co-localized with BrdU administered at E14 (Fig.29f,f',f''), but not at E12 (Fig.26g,g',g''). Consistently most neurons expressing Tbr1 co-localized with BrdU administered at E12 (Fig.29c,c',c'') but not with

BrdU administered at E14 (Fig.26d,d',d''). Most interestingly and in contrast to the majority of neurons that were randomly distributed throughout the cortex (Fig.29c'',d'',e'',f'',g''), a minor population formed a thin organised band at the most basal side of the Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex (Fig.29c',d'',e',f',g'). Moreover within this band neurons were organised in laminae, with those born at E14 localising above Tbr1 positive deep layer neurons, resembling the structure of cortical lamination as found in WT cortices (arrows in Fig.29d'). Thus, despite the severe alterations in cortical tissue organisation and the therefore aberrant environment, α-catenin deficient progenitor cells still generated different neuronal subtypes, even in their normal sequential order.

Taken together these data demonstrate that the deficiency of α-catenin impaired RG cells in their morphology, such that they lost their processes connecting to the apical and basal surface and it induced them to transiently accelerate their cell cycle. However both defects did not alter the precursor fate. Furthermore α-catenin deficient precursor cells gave rise to IPs and subsequently to neuronal subtypes of all cortical layers, suggesting that they maintained their intrinsic properties. This notion is further supported by the finding that AJs were still formed between Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortical cells and that molecules as Par3 and Prominin were concentrated in distinct membrane patches.

Figures

Figure 1:
Expression of Wnt ligands and canoncial Wnt signalling in the developing cortex:
Insitu hybridisation of Wnt ligands shows the expression of Wnt2b (a-c) and 3a (d-f) that is restricted to the cortical hem, localized laterally adjacent to the choroid plexus. Wnt7a was not detectable in the cortex at most early stages (g) and was expressed in the ventricular zone at E13 and E15 (h,i). Wnt 7b, firstly expressed in the precursor cells at E11 (k) became restricted to cortical neuronal layers during development (l,m). Both genes were additionally expressed in the ventricular zone of the ganglionic eminences (h-m). Wnt8b was only expressed in the medial cortex including the cortical hem and the hippocampus anlage at early stages (n) and was drawn back further medial, during development (o,p). Similar to Wnt8b, the canonical Wnt target Conductin was expressed in the medial cortex at early stages but became reduced till E15 (r-t). The Topgal reporter, detecting TCF/LEF dependent cell signalling showed activity throughout the cortex at E11 (u) but only in medial regions later in development (v,w).

Arrows indicate the cortical hem;
Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus;
Scale bars indicate 100μm;

Figure 1

Expression of Wnt ligands and canonical Wnt signalling in the developing cortex

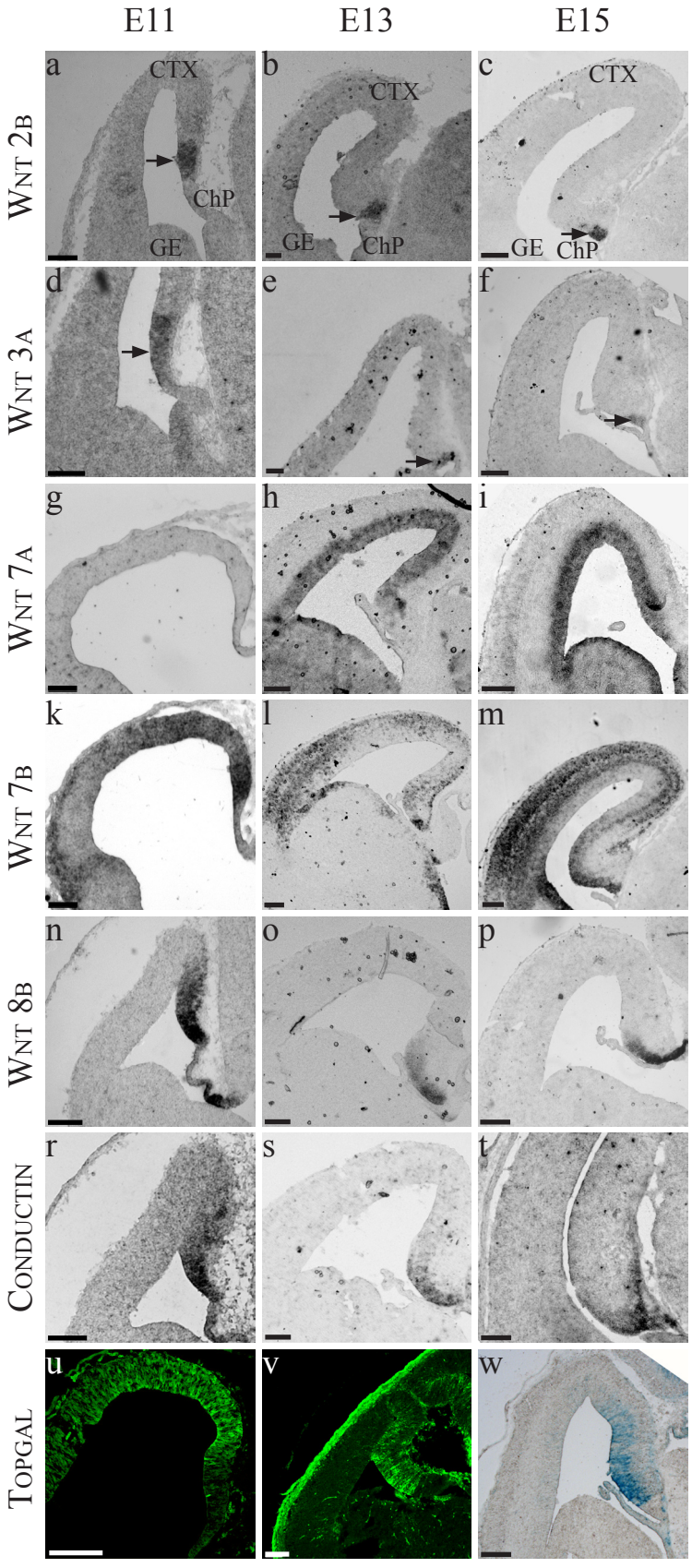


Figure 2

Deletion of β-catenin and canonical Wnt signalling by Emx1::Cre and hGFAP-Cre

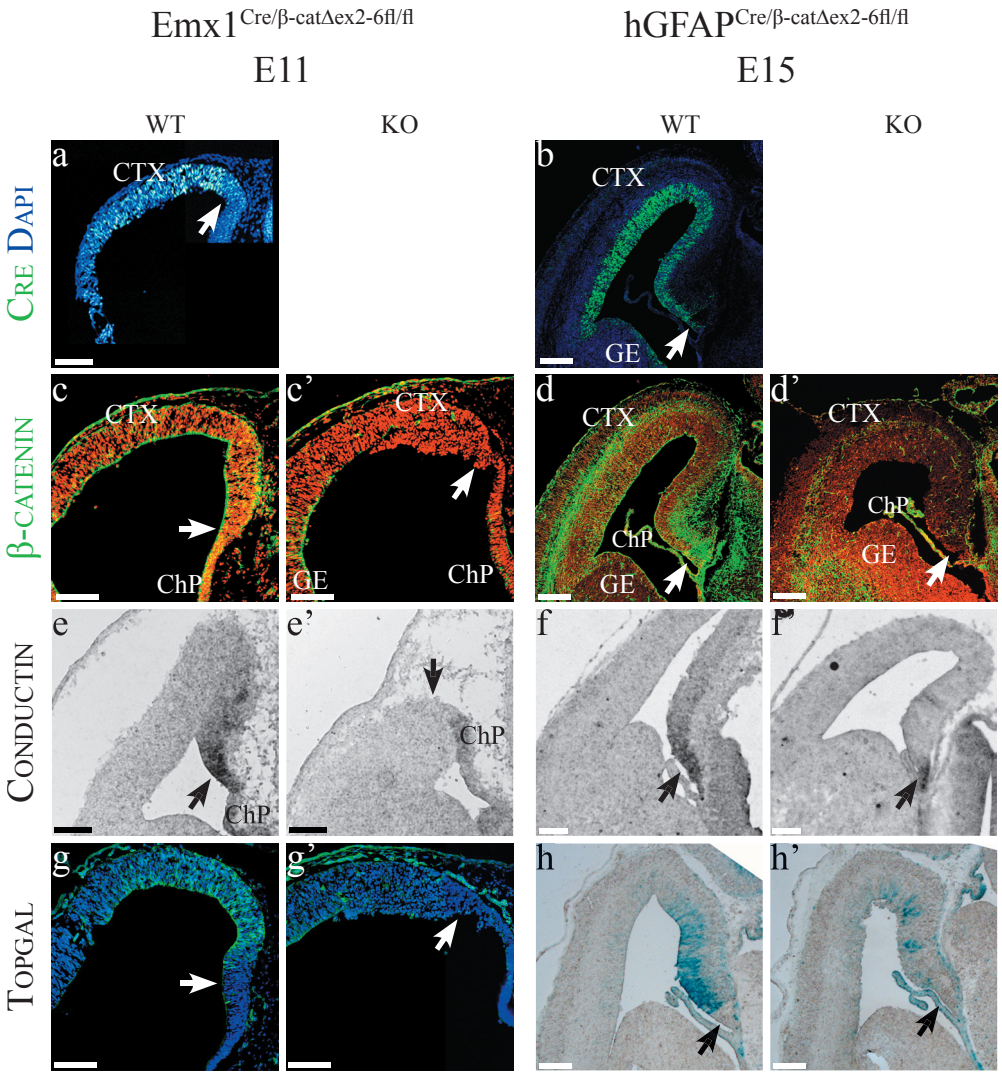


Figure 2

Deletion of β-catenin and canonical Wnt signalling by Emx1::Cre and hGFAP-Cre

Cre recombinase driven by the Emx1 promotor is expressed in the early cerebral cortex including the cortical hem (a) such that β-catenin is deleted from the cortex at E11 (c') whereas immunostaining for β-catenin in the WT (c) reveals a bright line along the ventricular surface. Expression of Conduction (e,e') and activity of the Topgal reporter (g,g') are lost in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant.

hGFAP-Cre is expressed in the ventricular zone of the entire cortex (b) and β-catenin is lost from the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex at E15 (d,d'). The expression of Conductin is almost abolished, apart from the most medial of the cortical hem (f,f'). Topgal reporter activity was strongly reduced in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex at E15 (h,h').

Arrows indicate the cortical hem;

Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus;

Scale bars indicate 100µm;

Figure 3

Cortical morphology in the absence of β-catenin

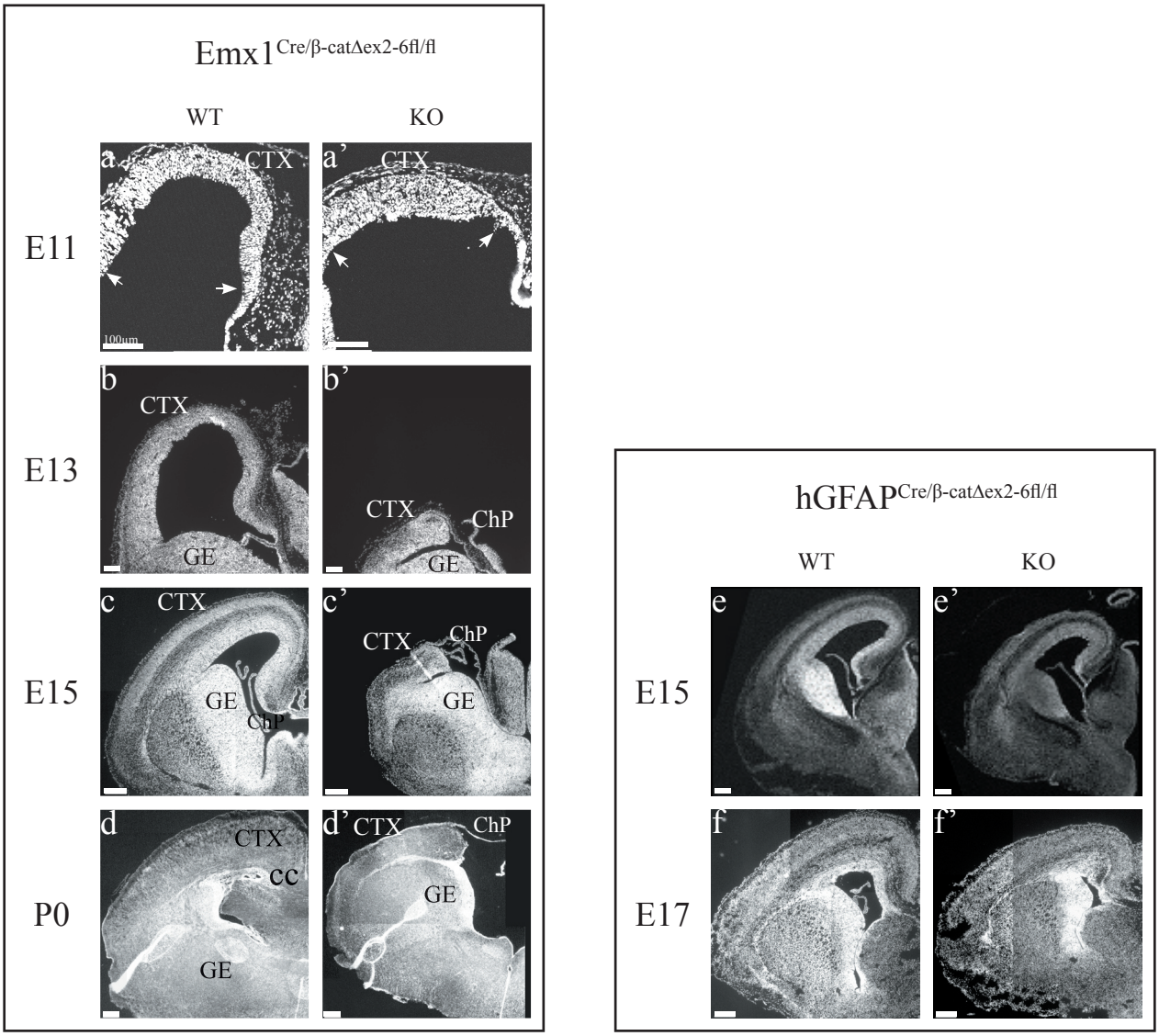


Figure 3

Cortical morphology in the absence of β-catenin

Cortical development in the presence and absence of β-catenin is demonstrated by cortical sections stained for nuclei (DAPI). The Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortex was already reduced in tangential expansion (a,a', indicated by arrows) and significantly smaller than wild type cortices at E13 (b,b'). In contrast to the wildtype, the Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortex hardly expanded from E13 to P0 whereas other regions of the brain developed normally (c,c',d,d'). These pictures also reveal the strongly impaired cortical architecture in the absence of β-catenin.

The cortical size was also reduced in the hGFAP$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortex compared to wildtype (e,e'), but less pronounced than the Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortex. Cortical architecture was better preserved than in the Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortex. The difference in size between the hGFAP$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ and wildtype cortex severed from E15 to E17 (f,f').

Arrows indicate the tangential expansion of the cortex;
Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus; CC: corpus callosum;
Scale bars indicate 100µm (a-c', e,e'); 200µm (d,d',f,f');

Figure 4

Cortical size and precursor proliferation in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant

The absence of β-catenin causes disturbed cortical architecture as visualized by immunostainings for Ki67 and β-III-tubulin depicting proliferating cells and neurons, respectively (a,a',b,b'). Neurons accumulate in the ventricular zone of the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (b'). Cortical organisation in respect to ventricular zone, subventricular zone and cortical plate is lost in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (a'). Despite this, cortical precursor cells are detected in both mutant cortices by Lhx2 (c-d') and Hes5 (e-e'), although strongly reduced in number. Immunostaining for PH3 reveals the disturbance of the mitotic pattern in the absence of β-catenin as mitotic figures are spread instead of localized at the ventricular surface and in the subventricular zone (g-h').

Quantitative analyses (i) revealed that cortical size and precursor proliferation is strongly reduced in the absence of β-catenin, however with a stronger effect upon early deletion of β-catenin during development (Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$). Note that cortical size and precursor proliferation is more reduced at caudal than at rostral levels.

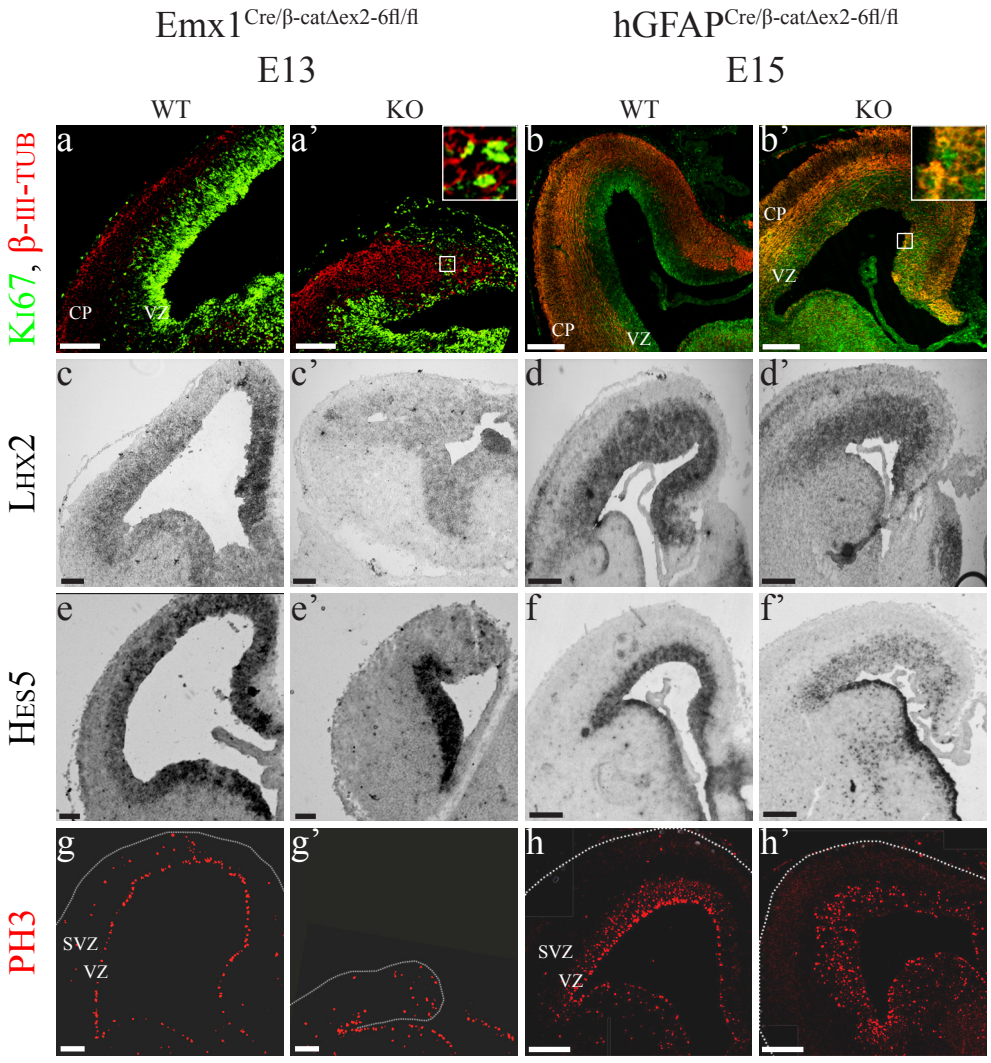
Abbreviations: VZ: ventricular zone, SVZ: subventricular zone, CP: cortical plate

Scale bars indicate 100μm

* indicate significance with * $p \leq 0{,}05$; ** $p \leq 0{,}01$

Figure 4

Cortical size and precursor proliferation in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ mutant



i

Quantitative analysis of cortical size and proliferation

(all valus given in % of WT)

cortical size	Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ E13	
rostral	53,9%	**
medial	38,8%	**
caudal	41,6%	**

cortical size	hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ E15	
rostral	108,7%	n.s
medial	81,8%	**
caudal	68,3%	*

PH3^{+}cells/ area	Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ E13	
rostral	51,4%	**
medial	63,1%	**
caudal	27,6%	**

PH3^{+}cells/ area	hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ E15	
rostral	88,0%	n.s
medial	69,7%	**
caudal	67,7%	*

Figure 5

Cell cycle exit, neurogenesis and cell death in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ at E11

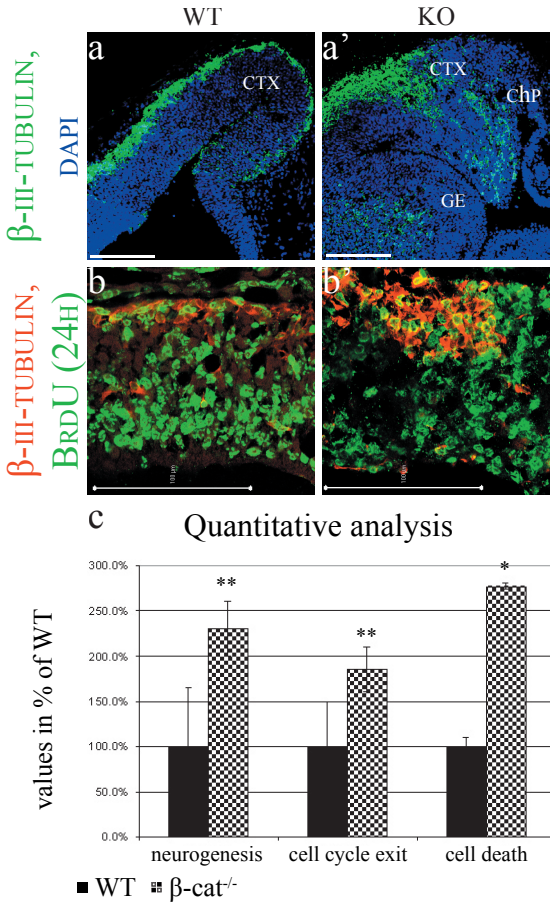


Figure 5
Cell cycle exit, neurogenesis and cell death in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex at E11
Immunostaining for β-III-tubulin shows a spread distributed of neurons in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex (a'). Quantitative analyses of neurogenesis revealed an increase of the number of neurons to more than 200% of WT levels (c). To examine the number of cells that left the cell cycle between E10 and E11 BrdU was administered at E10 and analysed together with β-III-tubulin at E11 (b), showing that it was almost doubled in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex compared to WT (b',c). Similar the number of apoptotic cells was strongly increased upon the loss of β-catenin (c).

Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus;
Scale bars indicate 100µm;
* indicate significance with * $p \leq 0{,}05$; ** $p \leq 0{,}01$;

Figure 6

Dorsal precursor cell identity in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ mutant cortices

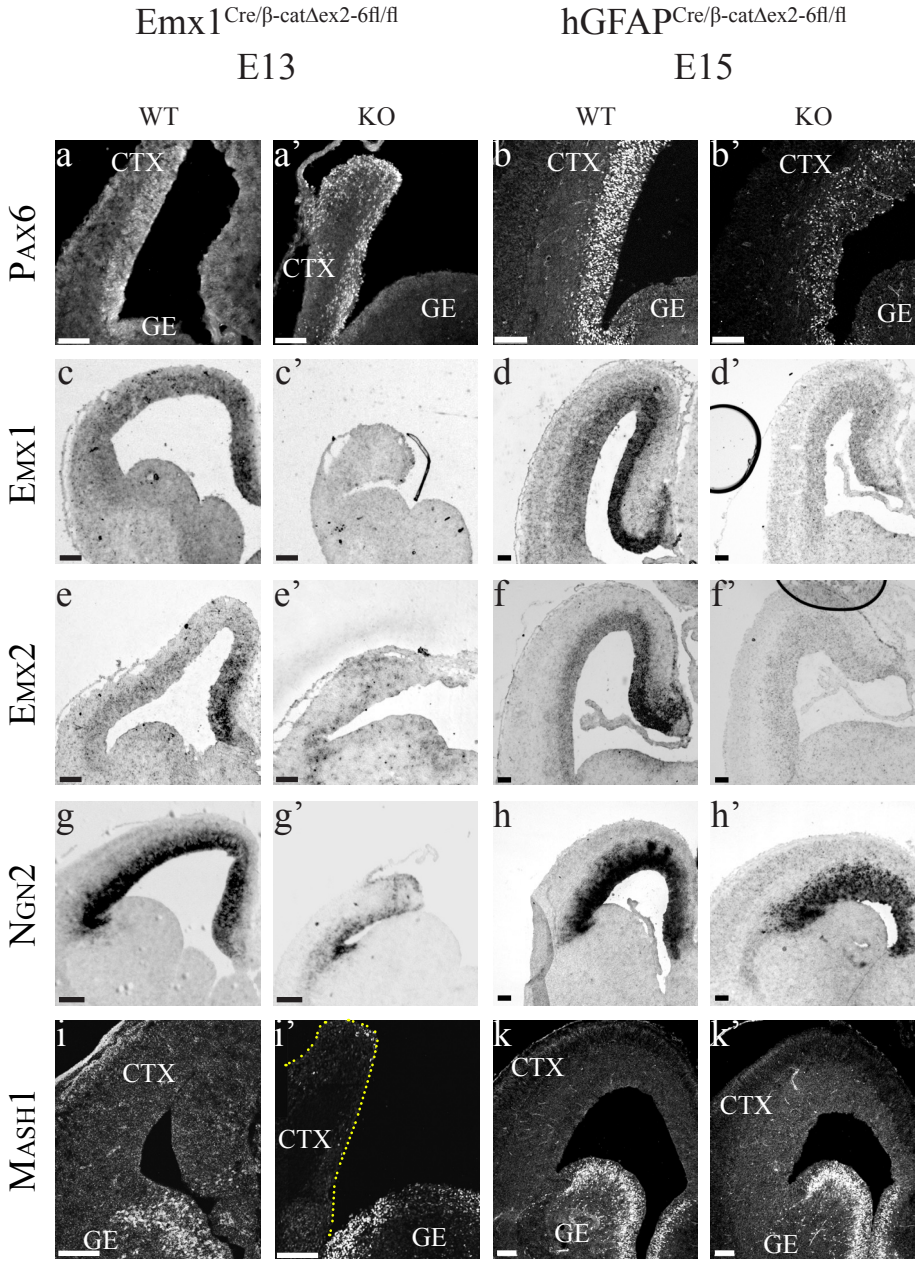


Figure 6

Dorsal precursor cell identity in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ mutant cortices

Cortical precursor cells were identified by immunostaining for Pax6 (a-b') and *insitu* hybridisation for Emx1 (c-d'), Emx2 (e-f') and Ngn2 (g-h'). Ventral precursor cells were identified by the expression of Mash1 (i-k'). These demonstrate that cortical precursor cells, although reduced in number, were correctly specified in the absence of β-catenin, expressing dorsal but not ventral transcription factors. Note that Emx1 (c-d') and Emx2 (e-f') are specifically reduced compared to Pax6 (a-b') and Ngn2 (g-h').

Dotted line indicates the cortex

Abbreviations: vz: ventricular zone, svz: subventricular zone, cp: cortical plate;

Scale bars indicate 100µm;

Figure 7

Canonical Wnt signalling in the cortical hem

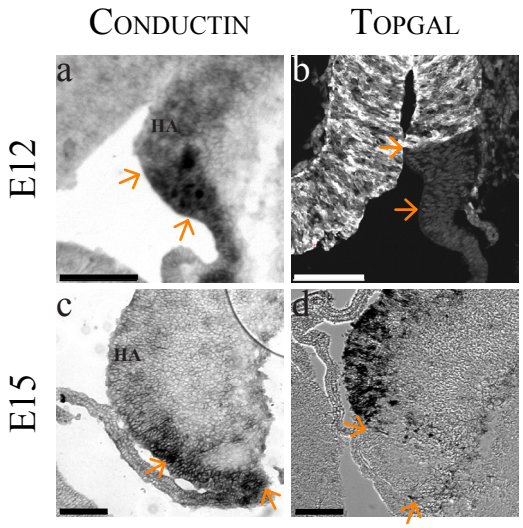


Figure 7
Canonical Wnt signalling in the cortical hem
Insitu hybridisation for the canonical Wnt target Conductin (a,c) that is expressed in the cortical hem and the adjacent hippocampus anlage at E12 and E15. The Topgal reporter in contrast is expressed in the hippocampus anlage but not in the cortical hem (b,d).

Arrows indicate the cortical hem;
Abbreviations: HA: hippocampus anlage;
Scale bars indicate 100µm;

Figure 8

Deletion of β-catenin by Nestin-Cre and loss of canonical Wnt signalling

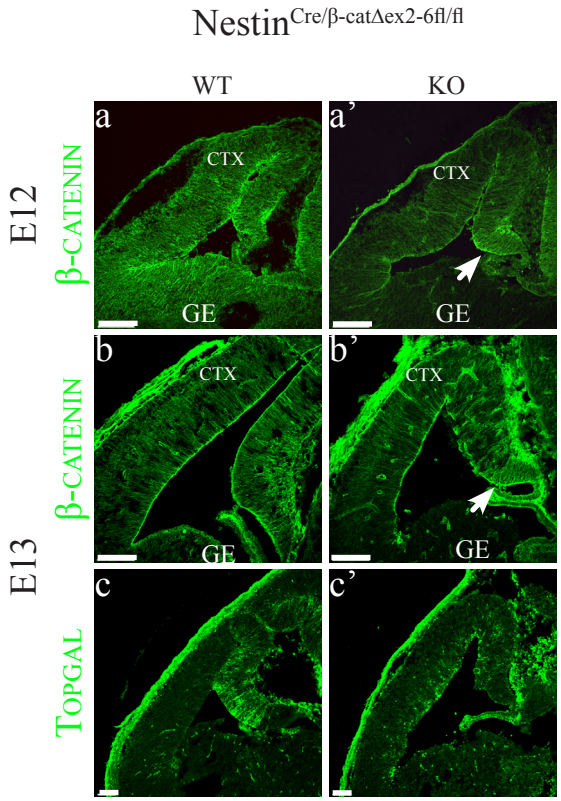


Figure 8
Deletion of β-catenin by Nestin-Cre and the loss of canonical Wnt signalling
β-catenin is deleted by Nestin-Cre from the ganglionic eminences and reduced in the cortex at E12 (a,a'). However recombination by this Cre line is particular slow such that β-catenin is still detectable in the E13 cortex (b,b'). Note the prominent immunostaining for β-catenin in the cortical hem where Nestin-Cre does not recombine (arrow in a',b'). Consistent with the reduced levels of β-catenin, Topgal reporter activity is strongly reduced in the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex at E13 (c,c').

Arrows indicate the cortical hem;
Abbreviations: CTX: cortex; GE: ganglionic eminences;
Scale bars indicate 100μm;

Figure 9

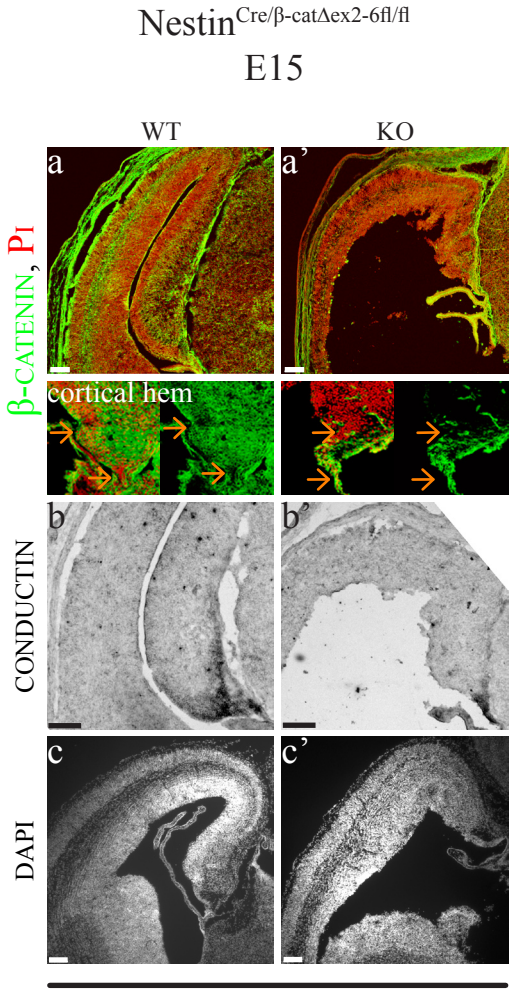
Cortical morphology of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant at E15

Immunostaining for β-catenin shows that the protein is completely absence from the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex but was not depleted from the cortical hem (a,a'). Conductin expression was absent from the medial mutant cortex but remained in the cortical hem (b,b'). Overview pictures of DAPI stained sections demonstrate the distinct morphology of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex compared to WT (c,c') but also compared to the former described β-catenin mutants (compare c' to Fig.3). Quantitative analyses of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex revealed that is was reduced in size at caudal levels (d) but precursor proliferation was only weakly reduced compared to the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants (compare d to Fig.4i)

Arrows indicate the cortical hem;
Scale bars indicate 100µm;

Figure 9

Cortical morphology in the $Nestin^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant at E15



d

Quantitative analysis

(all valus given in % of WT)

cortical size	$Nestin^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ E15	
rostral	117,8%	n.s
medial	110,3%	*
caudal	78,2%	**

$PH3^{+}$cells/ area	$Nestin^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ E15	
rostral	101,6%	n.s
medial	70,8%	**
caudal	89,3%	n.s

Figure 10

Proliferation and Neurogenesis in the Nestin$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex

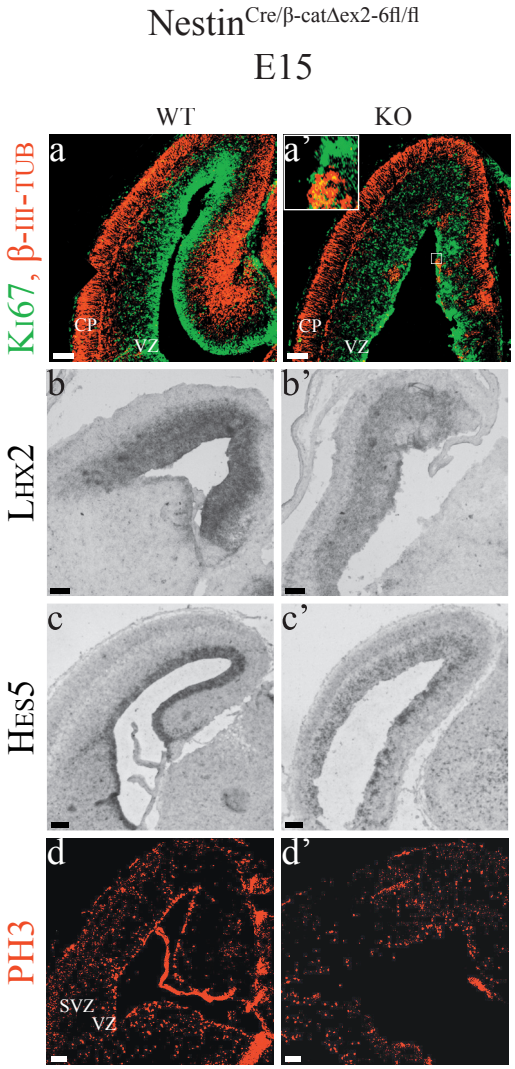


Figure 10

Proliferation and Neurogenesis in the Nestin$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ mutant

Immunostaining for Ki67 depicting proliferating precursor cells and β-III-tubulin depicting neurons (a,a'), demonstrates that in the Nestin$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex neurons are present in the ventricular zone, similar to the hGFAP$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex although in less numbers (compare a' to Fig.4b'). *Insitu* hybridisation for Lhx2 and Hes5 and immunostaining for PH3 visualize the distribution of precursor cells in WT and the Nestin$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex (b-d').

Abbreviations: VZ: ventricular zone, SVZ: subventricular zone, CP: cortical plate;
Scale bars indicate 100µm;

Figure 11

Cortical precursor cell identity in the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

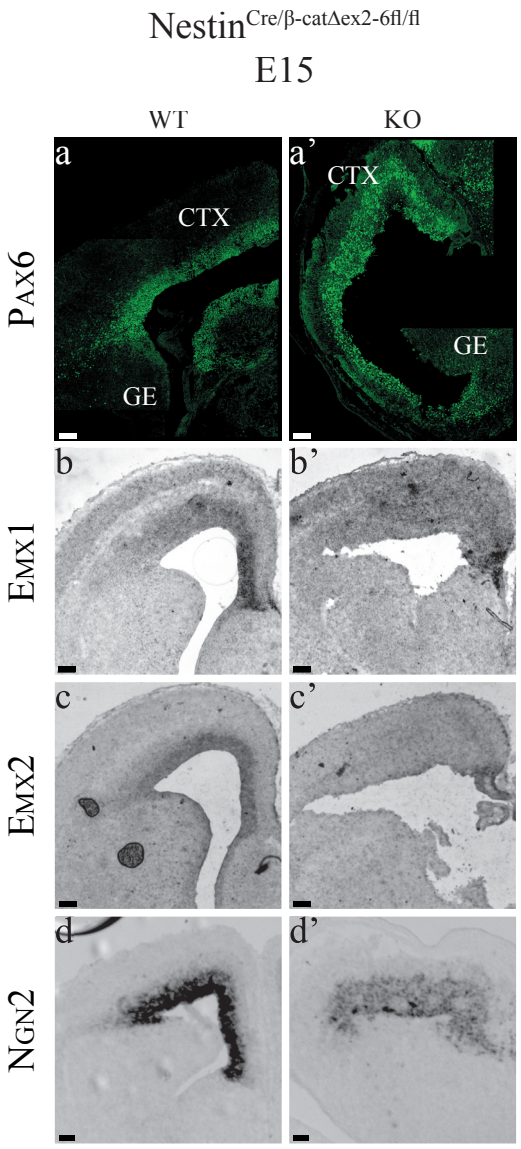


Figure 11
Cortical precursor cell identity in the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex
Immunostaining for Pax6 (a,a') and *insitu* hybridisation for Emx1 (b,b'), Emx2 (c,c') and Ngn2 (d,d') revealed the dorsal character of precursor cells in the WT and Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. Note that the ventricular zone of the Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex is strongly expanded as revealed by staining for Pax6 (a').

Abbreviations: CTX: cortex; GE: ganglionic eminences;
Scale bars indicate 100µm;

Figure 12

Gene expression within the cortical hem

Cells of cortical hem identity were identified by the expression of Wnt2b (a-c') and Lmx1a (d-f') that was observed in all three β-catenin mutants (a',b',c',d',e',f'), localized adjacent to the choroid plexus similar to WT (a,b,c,d,e,f). Wnt3a expression was not detected in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (g,g') in contrast to the $hGFAP^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ (h,h') and $Nestin^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (i,i'). Expression of Wnt8b (k,l,m) and Id3 (n,o,p) both expressed in the cortical hem and the medial cortex in WT were reduced in the three β-catenin mutants (k',l',m',n',o',p'). In the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex Wnt8b (k') and Id3 (n') were only expressed adjacent to the choroid plexus supposedly in the cortical hem. The expression of BMP6 within the cortical hem was lost in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ (q,q') and $hGFAP^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ (r,r') but not in the $Nestin^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (s,s').

Arrows are indicating the cortical hem;
Abbreviation: ChP: choroid plexus;
Scale bars indicate 100µm;

Figure 12

Gene expression within the cortical hem

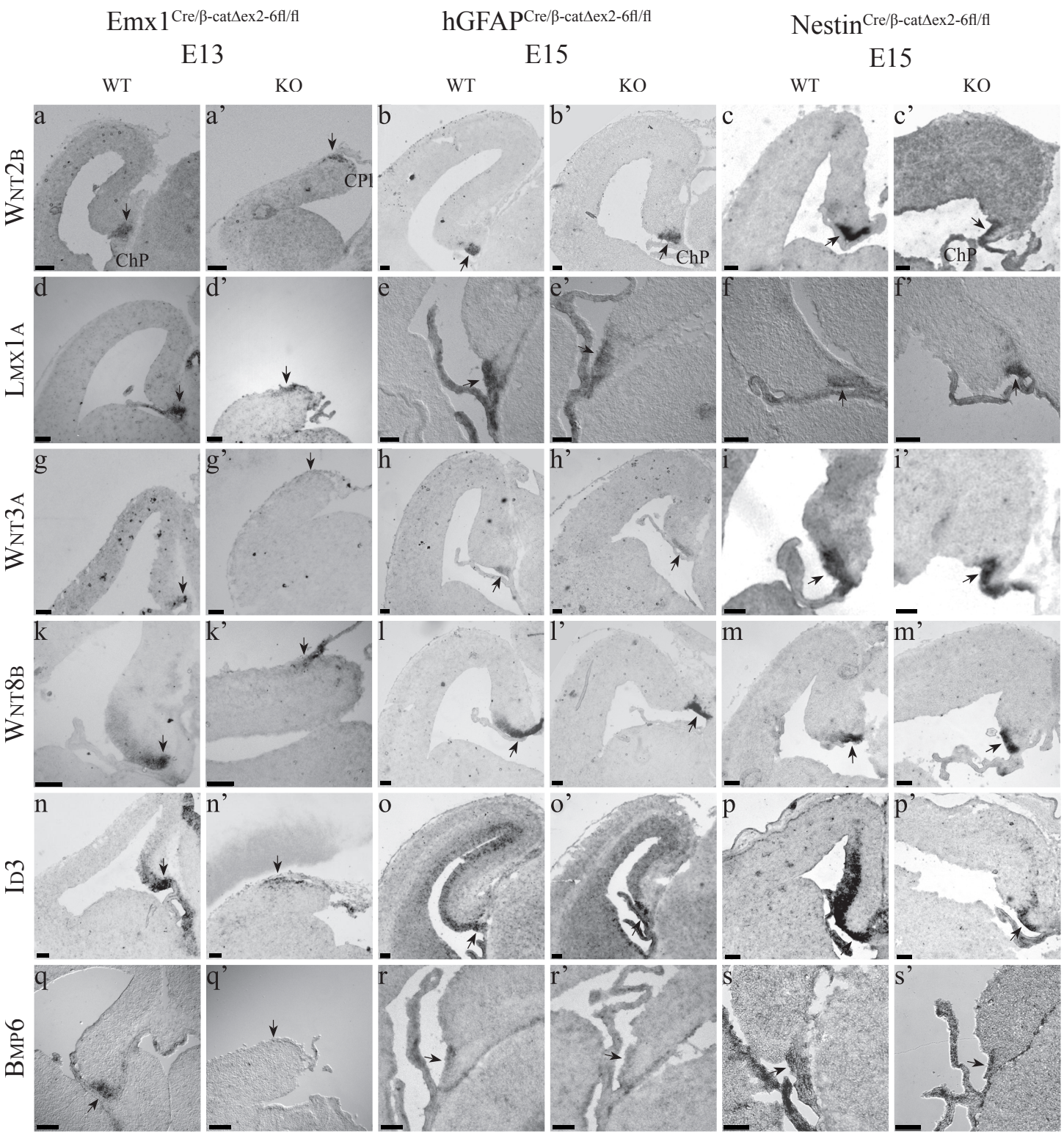


Figure 13

Precursor identity in the β-catenin deficient cortex – subventricular zone progenitors

Intermediate progenitor cells were identified by their expression of Svet1 (a-c'), Cux2 (d-f'), NeudoD (g-g') and Tbr2 (k-m'). Intermediate progenitor cells were present in all three β-catenin mutants although not restricted to the subventricular zone in their localisation. Double staining of Pax6 and Tbr2 revealed the populations of radial glial and intermediate progenitor cells, respectively (n,n'). Quantitative analysis of these demonstrated that the relative size of these populations was not changed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex (o,o').

Abbreviations: vz: ventricular zone, svz: subventricular zone, cp: cortical plate;
Scale bars indicate 100μm;

Figure 13

Precursor Identity in the β-catenin-deficient cortex - subventricular zone progenitors

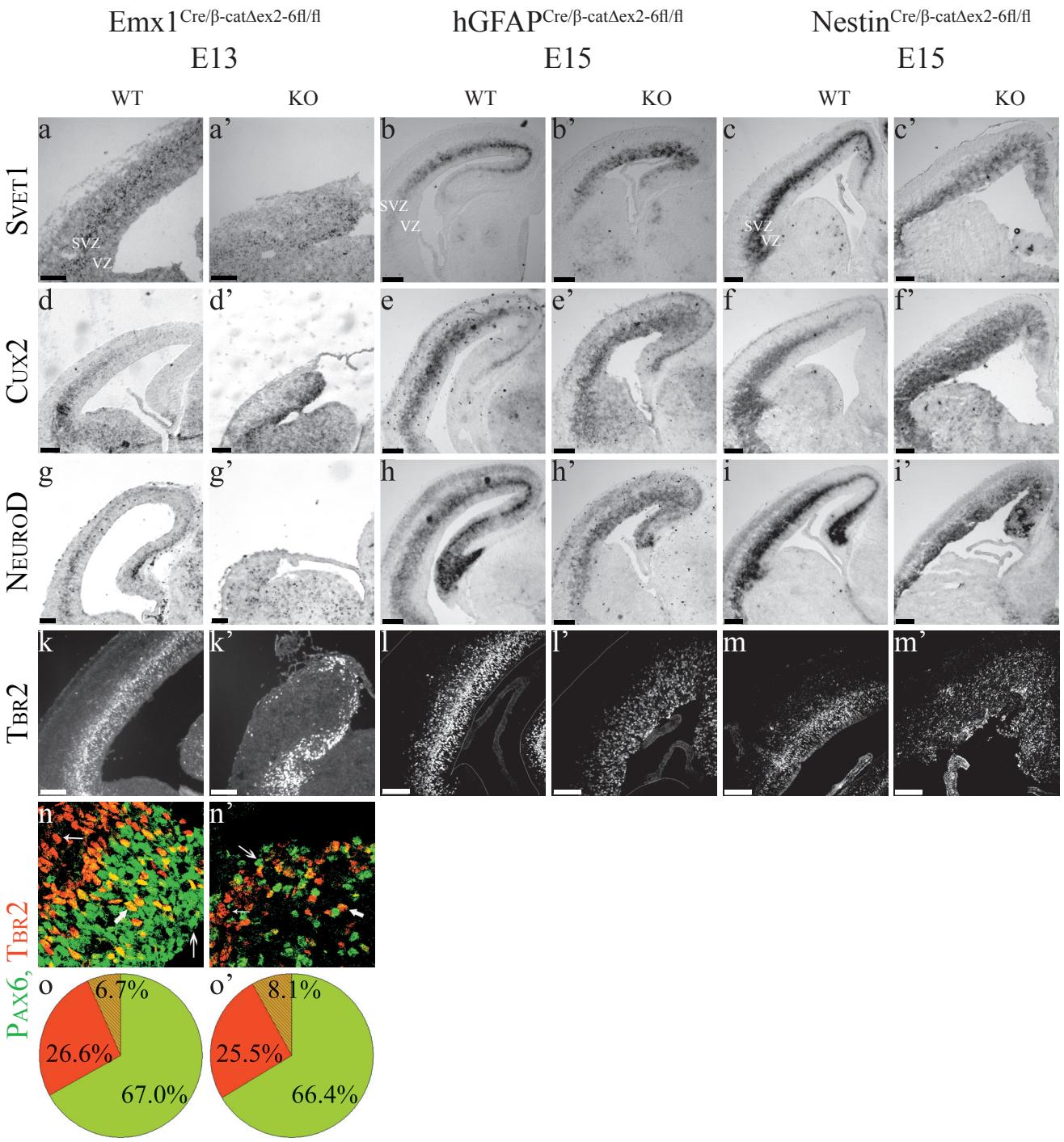


Figure 14

Generation of cortical neuronal subtypes in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ mutant

Distinct neuronal subtypes were identified by their respective gene expression. Neurons for layer I were immunostained for Reelin (a) and Calretinin (b). *Insitu* hybridisation for Cux2 (c) and Satb2 (d) depicted neurons of layer II and III. Neurons of layer V and VI express Er81 (e) and Tbr1 (f) respectively. Neurons of all layers were present in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex (a',b',c',d',e',f') although not organized in laminae as in WT (a,b,c,d,e,f). Astroglial cells, identified by immunostaining for GFAP were present in WT and in the Emx1$^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex but displayed an impaired morphology (enlarged details in g,g').

Dotted lines indicate the border between the cortex and the gangionic eminences;
Abbreviations: CTX: cortex; GE: ganglionic eminences; IZ: intermediate zone;
Scale bars indicate 10µm (a-b'); 100µm (c-g');

Figure 14

Generation of cortical neuronal subtypes in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant

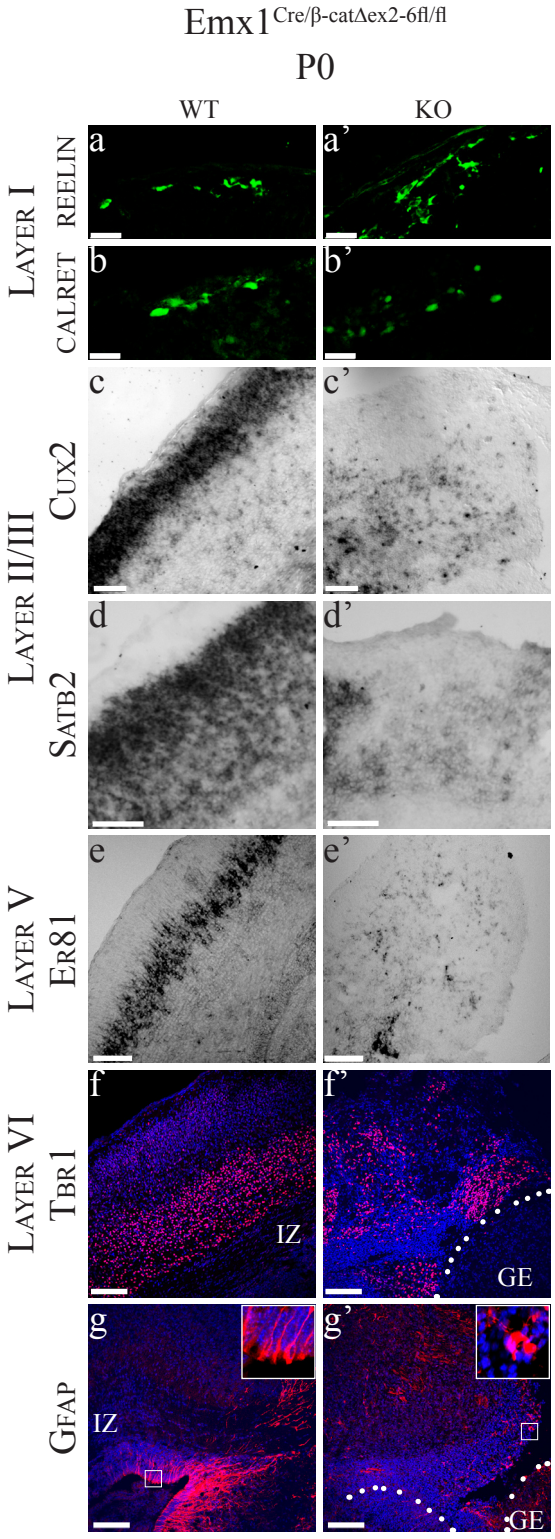


Figure 15

BrdU-birthdating of cortical neurons in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant

BrdU was applied at E11 (a-c') or E16 (d-f') to pregnant mice to designate the neurons generated at the respective developmental stages. Double-staining with neuronal markers was used to reveal the identity of these neurons. In WT cortex, neurons generated at E11 mostly expressed Tbr1 (c) but not Cux2 (a) or Er81 (b), whereas neurons generated at E16 co-localized with Cux (d) but not Er81 (e) or Tbr1 (f). In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex in contrast neurons that had been generated at E11 were found to co-localize with Tbr1 (c') but also with Cux2 (a'), and those generated at E16 expressed Cux2 (d') as well as Er81 (e') and Tbr1 (f').

White arrows indicate cells only positive for BrdU;
Orange arrows indicate cells positive for BrdU and the respective neuronal marker;
Scale bars indicate 100μm;

Figure 15

BrdU-birthdating of cortical neurons in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant

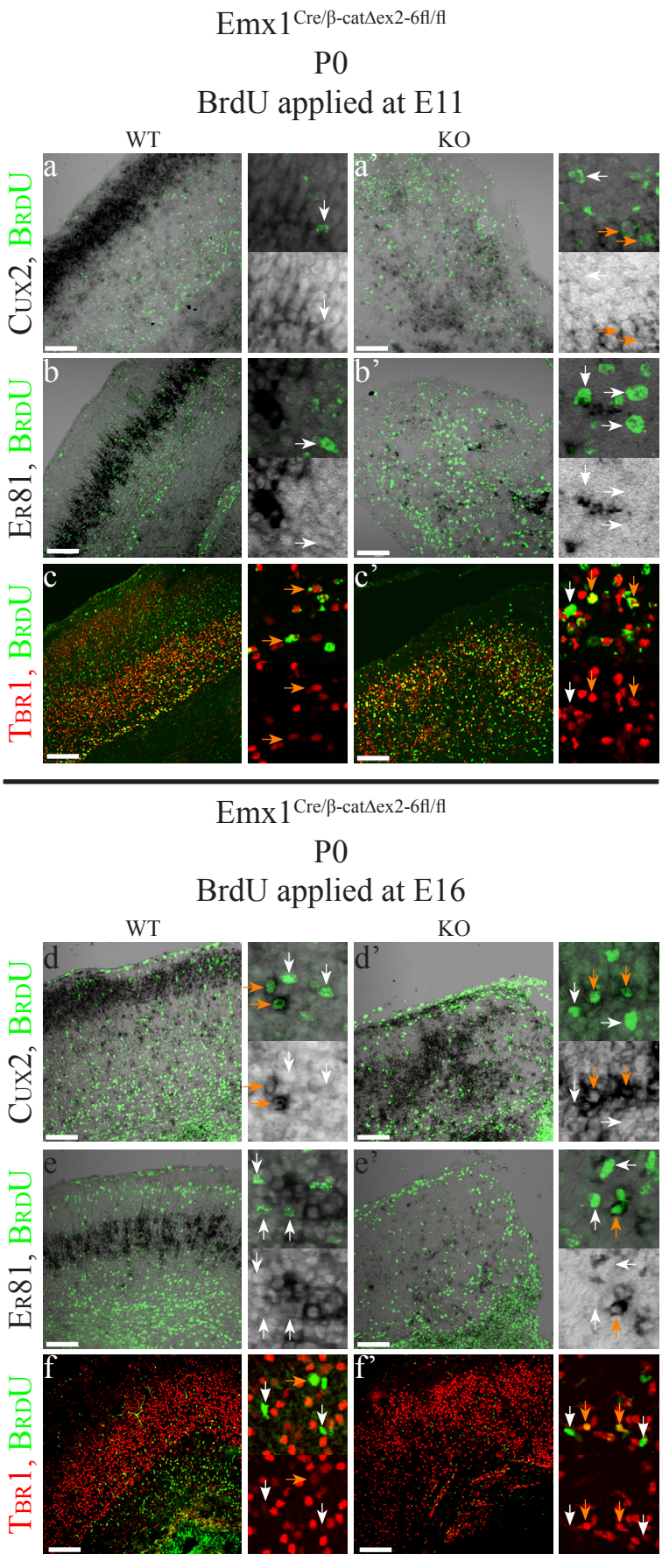


Figure 16

Deletion of β- and α-catenin by Emx1::Cre

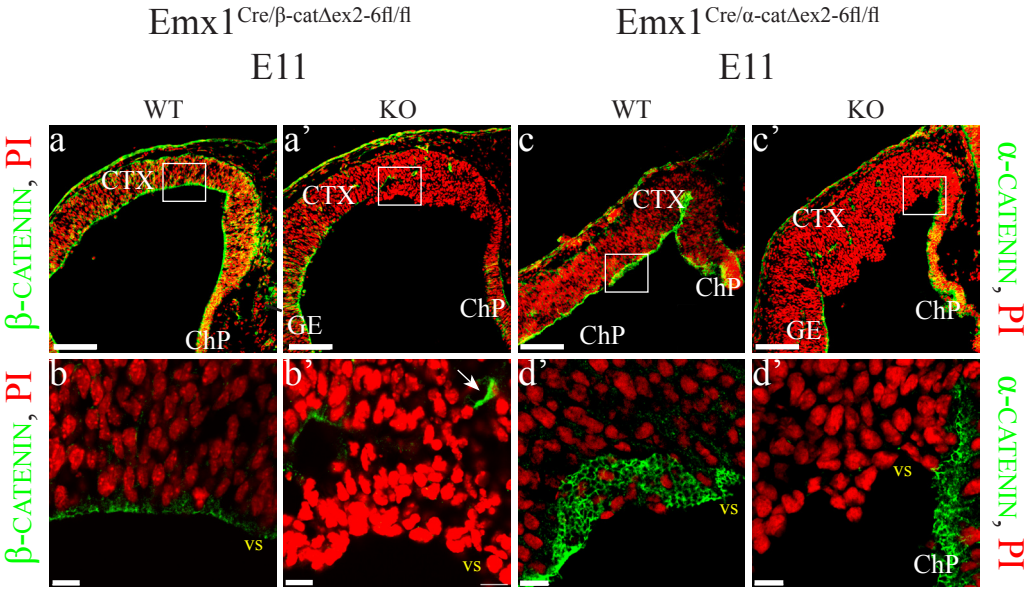


Figure 16
Deletion of β- and α-catenin by Emx1::Cre
Immunostaining for β-catenin (a,b) and α-catenin (c,d) shows the localisation of the proteins along the ventricular surface in the WT cortex. α-catenin (c'd') and β-catenin (a',b') protein is lost in the respective mutants at E11 but is still present in the ganglionic eminences, the choroid plexus, and in blood vessels where Emx1::Cre is not expressed.

Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus;
Scale bars indicate 10µm (b,b',d,d'); 100µm (a,a',c,c');

Figure 17

Adherens Junctions in the absence of β-catenin and α-catenin at E11

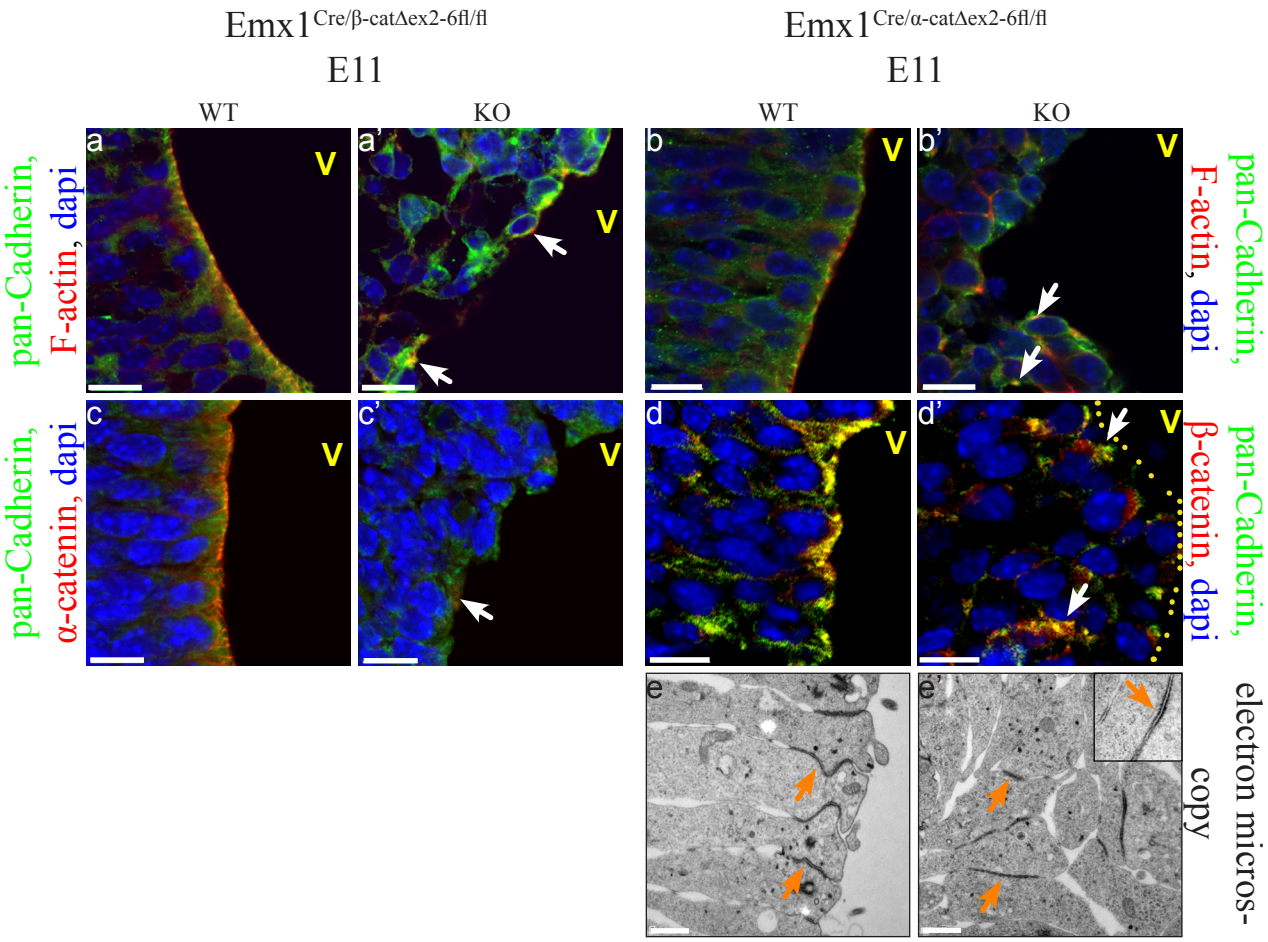


Figure 17

Adherens junctions in the absence of β-catenin and α-catenin at E11

α-catenin (c), β-catenin (d) and cadherins (a,b), participating in the formation of adherens junctions, were visualized by immunostaining and localized at the ventricular surface in WT (a,b,c,d). Factin was detected by using rhodamine labelled phalloidin (a,b). These stainings revealed that cadherins, F-actin and α-catenin localized in close proximity to each other in Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ cortical cells (a',c'). Reciprocally cadherins, F-actin and β-catenin were localized in close proximity to each other in the Emx1$^{Cre/α\text{-}catΔex2fl/fl}$ cortical cells (b',d'). Note that adherens junction proteins did not absolutely co-localise in the catenin mutant cells but were often concentrated at one side of the cells (arrows in a',b',c',d'). Adherens junctions were displayed by electron microscopy as long electron dense structures (indicated by orange arrows in e) oriented perpendicular to the ventricular surface. Similar structures were observed between Emx1$^{Cre/α\text{-}catΔex2fl/fl}$ cortical cells (e') indicating that α-catenin deficient cells maintain cell-cell contacts similar to adherens junctions.

White arrows indicate patches of co-localisation;
Orange arrows indicate adherens junctions;
Abbreviations: v: ventricular lumen;
Scale bars indicate 10μm (a-d'); 100nm (e,e');

Figure 18

Adherens Junctions in the absence of β-catenin and α-catenin at E13

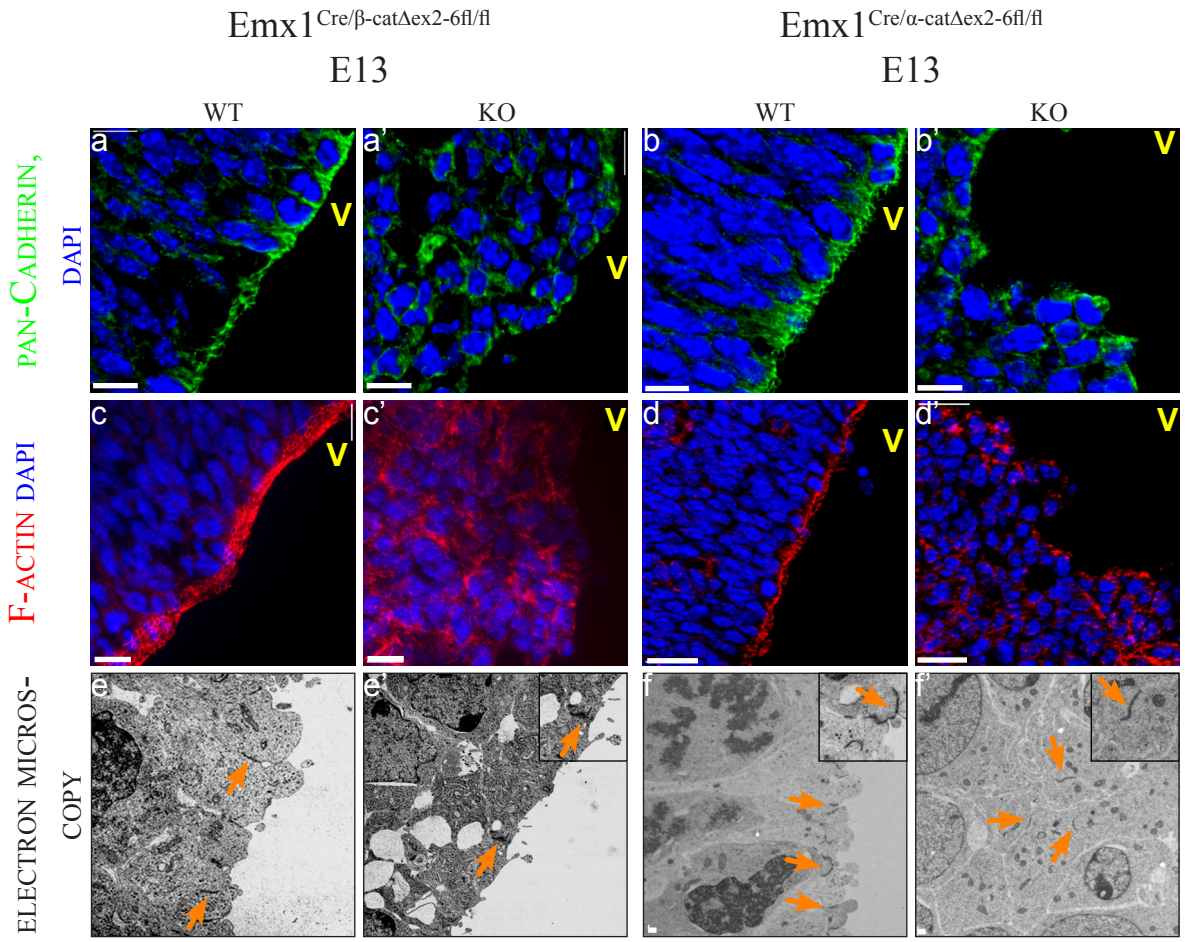


Figure 18

Adherens junctions in the absence of β-catenin and α-catenin at E13

Cadherin proteins (a-b') and Factin (c-d') were detected by immunostaining and rhodamine labelled phalloidin respectively. Both molecules localize to the ventricular surface in the WT cortex (a,b,c,d) but become redistributed in the absence of β-catenin (a',c') or α-catenin (b',d'). Electron microscopy revealed that cell-cell contacts reminiscent of adherens junctions were present in the Emx1$^{Cre/β\text{-}catΔex2\text{-}6fl/fl}$ and the Emx1$^{Cre/α\text{-}catΔex2fl/fl}$ cortical cells although found not only localized near the ventricular lumen but also within the cortical parenchyma (e',f').

Orange arrows indicate adherens junctions;
Abbreviations: v: ventricular lumen;
Scale bars indicate 10µm (a-d'); 500nm (f,f');

Figure 19

Morphology of radial glial cells lacking β-catenin or α-catenin

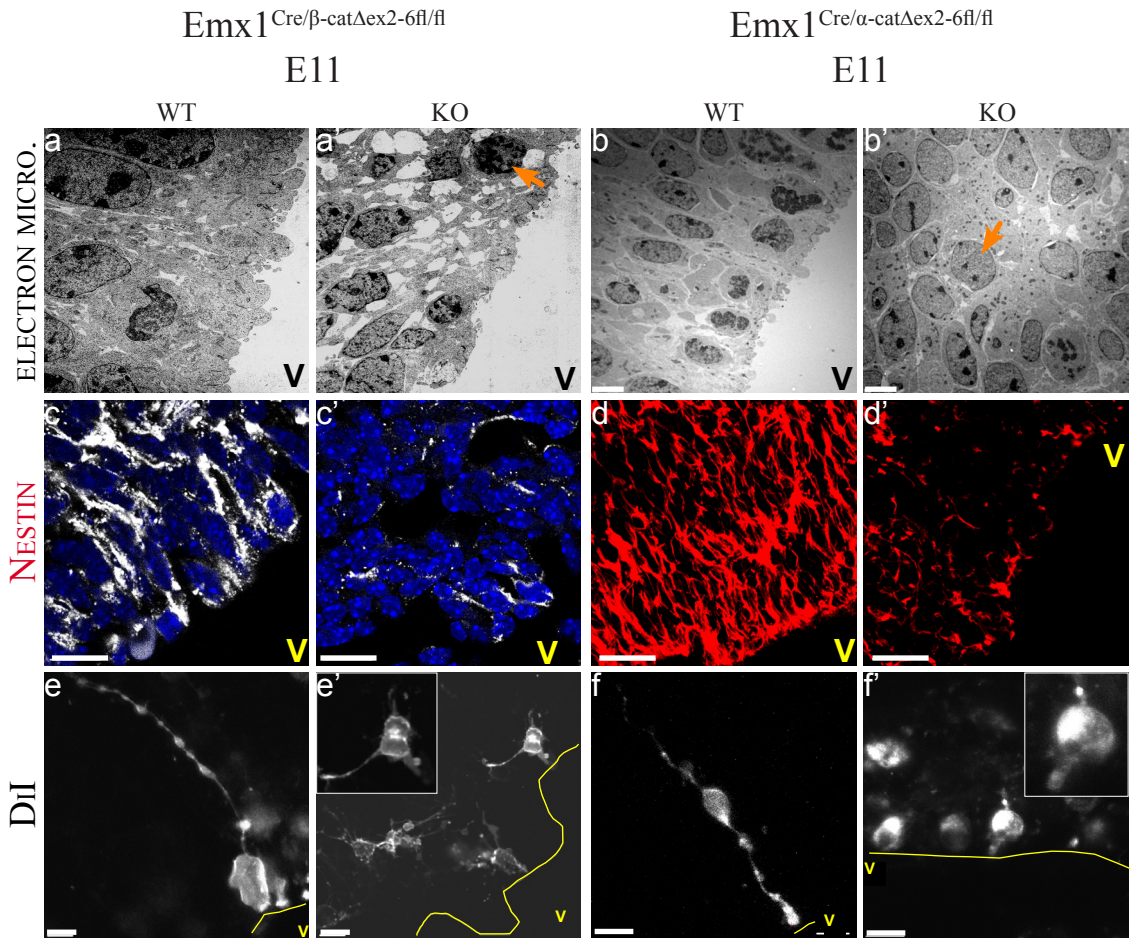


Figure 19

Morphology of radial glial cells lacking β-catenin or α-catenin

β- or α-catenin deficient cortical cells display a round instead of elongated morphology and round shaped nuclei, as revealed by electron microscopy (a-b'). This was consistent with the reduced number of radial processes visualized by immunostaining for the intermediate filament Nestin (c-d'). Single cortical cells were labelled from the ventricular surface by DiI revealing the long, bipolar radial processes of WT radial glial cells (e,f). β-catenin and α-catenin deficient cells in contrast show multiple short processes (e',f').

Orange arrows indicate round shaped nuclei;
Abbreviations: v: ventricular lumen;
Scale bars indicate 5µm (b,b',e,e',f,f'); 10µm (c,c',d,d');

Figure 20

Distribution of apical molecules in β-catenin and α-catenin deficient cortices

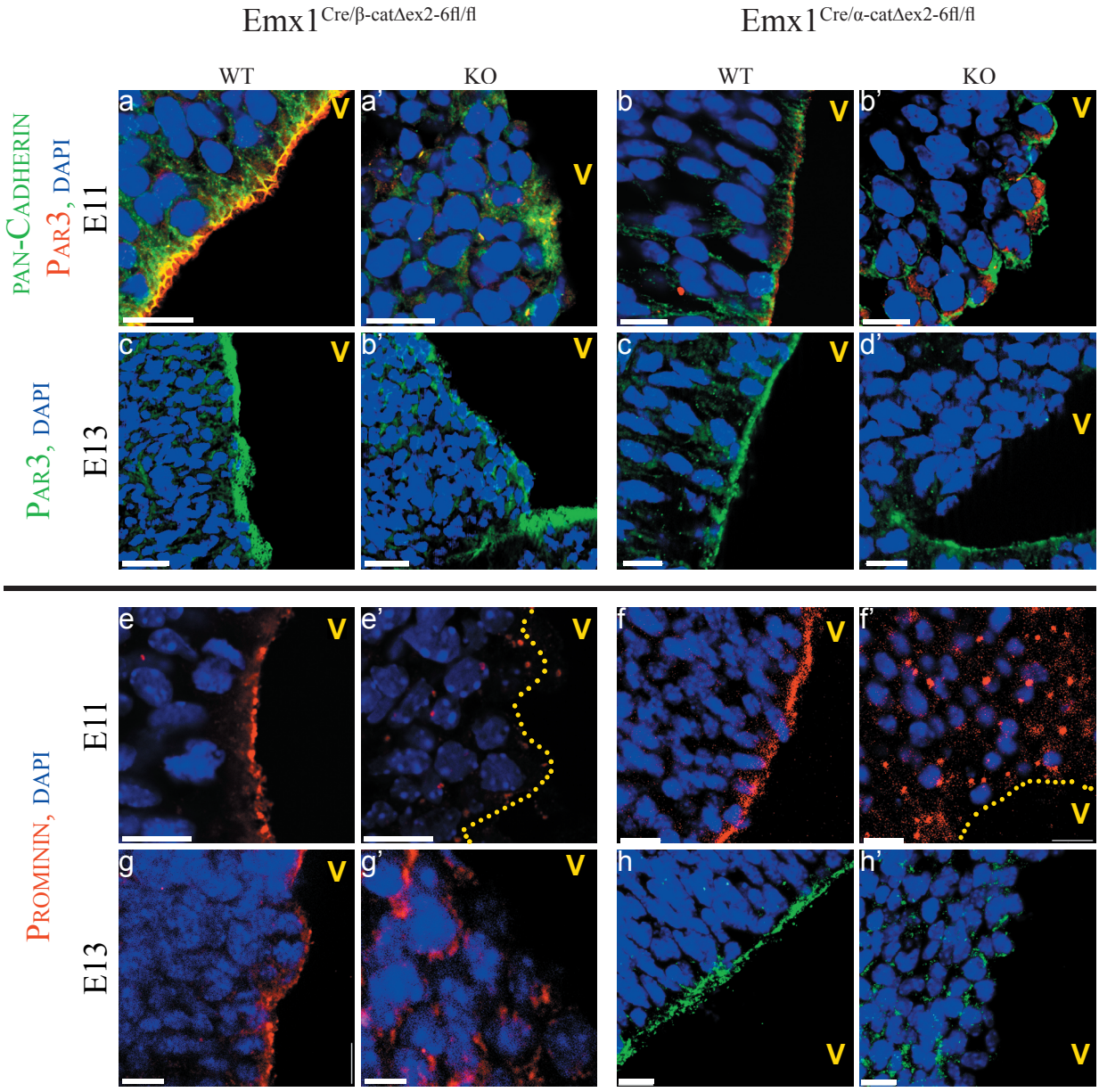


Figure 20

Distribution of apical molecules in the β-catenin and α-catenin deficient cortices

Immunostaining for Par3 reveals the localisation of this molecule along the ventricular lumen of WT cortical cells (a,b,c,d), colocalizing with cadherin, depicting the adherens junctions (a,c), at E11 (a,b) and at E13 (c,d). Immunostaining for Prominin shows the localization of this molecule at the apical surface of WT cortical cells (e-h). Par3 and Prominin are present but redistributed in the catenin mutant cells at E11 (a',b',e',f') and at E13 (c',d',g',h').

Dotted lines outline the cortex;
Abbreviations: v: ventricular lumen;
Scale bars indicate 10µm;

Figure 21:

Western Blot analysis of Par-complex molecules in the Emx1$^{Cre/\alpha\text{-}catΔex2\text{-}6fl/fl}$ cortex

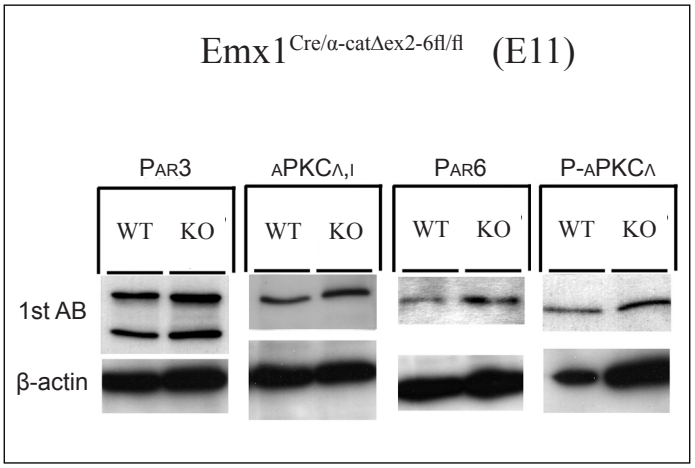


Figure 21

Western Blot analysis of Par-complex molecules in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex

Proteins participating in the formation of the Par complex were analysed by Western Blot revealing that similar amounts of Par3 (150, 180kb), aPKCλ,ι (74kb) and Par6 (41kb) are present in WT and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortices. Additionally aPKC is present in its phosphorylated, active form in WT and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex.

Abbreviations: AB: antibody

Figure 22

Morphology of the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

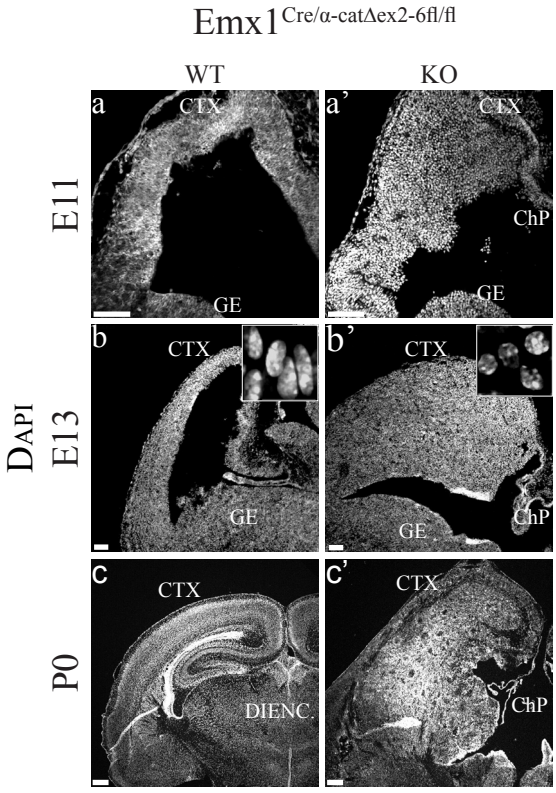


Figure 22

Morphology of the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex

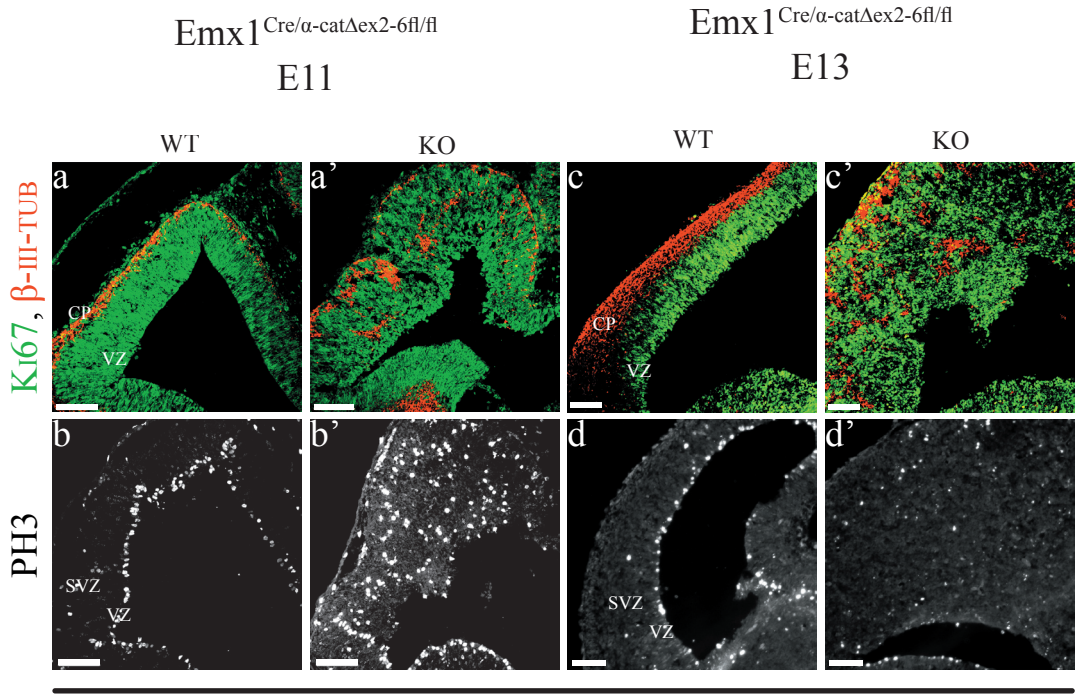
Cortical development in the presence and absence of α-catenin is demonstrated by cortical sections stained for nuclei (DAPI). The Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex is already increased at E11 compared to WT(a,a') and becomes further enlarged during development (b-c'). Cortical morphology is strongly impaired in the absence of α-catenin such that the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex does not develop in laminae. Nuclei in the mutant cortex display a round insteasd of elongated shape (details in b and b').

Abbreviations: CTX: cortex; GE: ganglionic eminences; ChP: choroid plexus; DIENC: diencephalon

Scale bars indicate 100µm;

Figure 23

Proliferation and neurogenesis and cell cycle exit in the Emx1$^{Cre/\alpha\text{-}catΔex2\text{-}6fl/fl}$ cortex



e

Quantitative analysis of cortical size, proliferation and cell cycle exit

(all valus given in % of WT)

Emx1$^{Cre/\alpha\text{-}catΔex2\text{-}6fl/fl}$ E11		
cortical size	152,6%	**
PH3^{+}cells / area	115%	**
β-III-tub.$^{+}$ cells / area	60,1%	*
Labelling Index	171,4%	**
cell death	117,4%	n.s

Emx1$^{Cre/\alpha\text{-}catΔex2\text{-}6fl/fl}$ E13			
cortical size	rostral	277,2%	**
	medial	258,9%	**
	caudal	195,3%	**
Ph3^{+}cells/area	rostral	113,5%	n.s
	medial	115,4%	n.s
	caudal	95,6%	n.s

Figure 23

Proliferation, neuogenesis and cell cycle exit in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex

Immunostaining for Ki67 and β-III-tubulin indicated proliferating precursor cells and neurons respectively, demonstrating that disorganisation of the ventricular zone and the cortical plate in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex at E11 (a,a') and E13 (c,c'). Immunostaining for PH3 depicts mitotic cells that are localized at the ventricular surface and in the subventricular zone in the WT cortex (b,d) but are randomly distributed in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex (b',d'). Quantitative analysis revealed that at E11 the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex is increase in size, displays an increased number of mitotic cells but fewer neurons compare to WT (e). The highly increased labelling index indicates that α-catenin deficient precursor cells display a shorter cell cycle than WT cells (e). Cell death was not significantly changed upon the loss of α-catenin. Although cortical size is strongly increased in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex compared to WT, with the most prominent increase at rostral levels, the number of mitotic cells per area is similar between WT and Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex at E13 (e).

Abbreviations: VZ: ventricular zone, SVZ: subventricular zone, CP: cortical plate; n.s: not significant;
Scale bars indicate 100µm
* indicate significance with * $p \leq 0,05$; ** $p \leq 0,01$

Figure 24

Sonic hedgehog signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

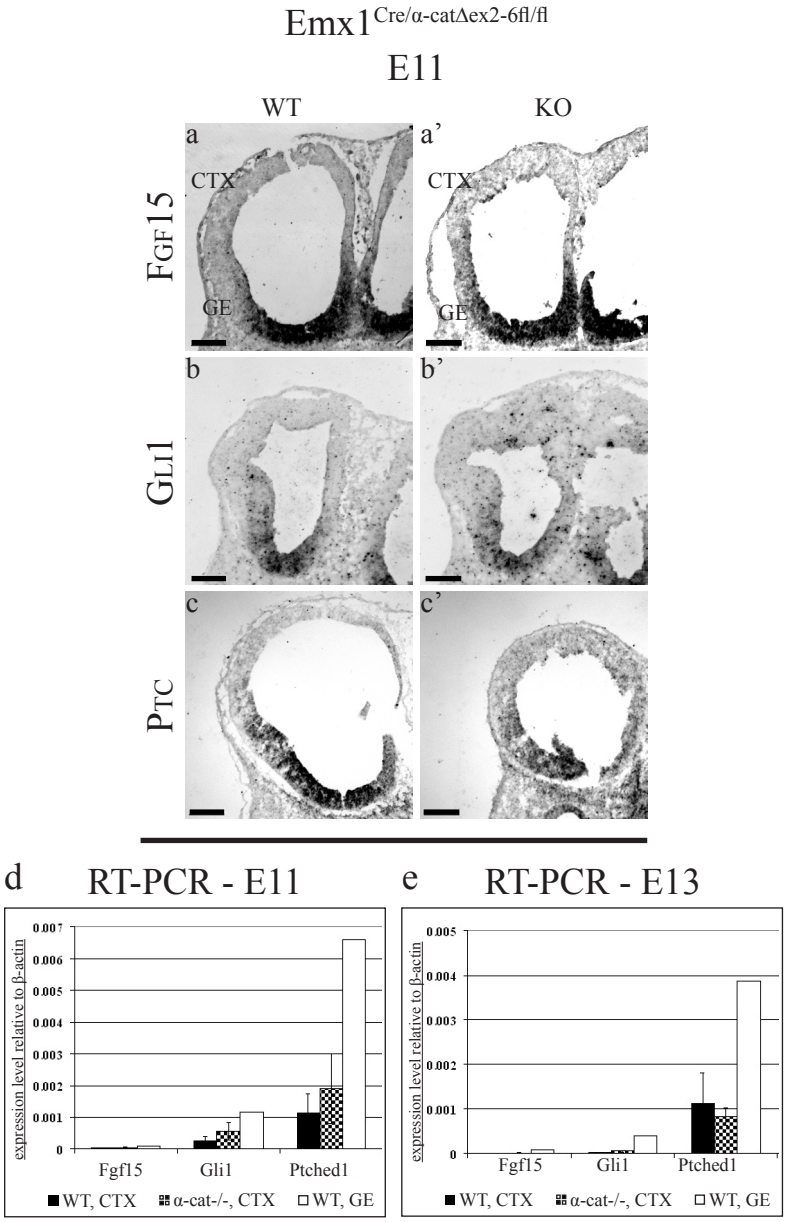


Figure 24

Sonic hedgehog signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex

Insitu hybridisation for the sonic hedgehog signalling targets Fgf15 (a,a'), Gli1 (b,b') and Ptc (c,c') revealed that these genes are highly expressed in the ganglionic eminences at E11 but not in the cortex of WT as well as Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutants. RT-PCR for these genes confirmed the results from *insitu* hybridisation at E11 (d) and at E13 (e).

Abbreviations: CTX: cortex; GE: ganglionic eminences;
Scale bars indicate 100µm;

Figure 25

MAP-kinase signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

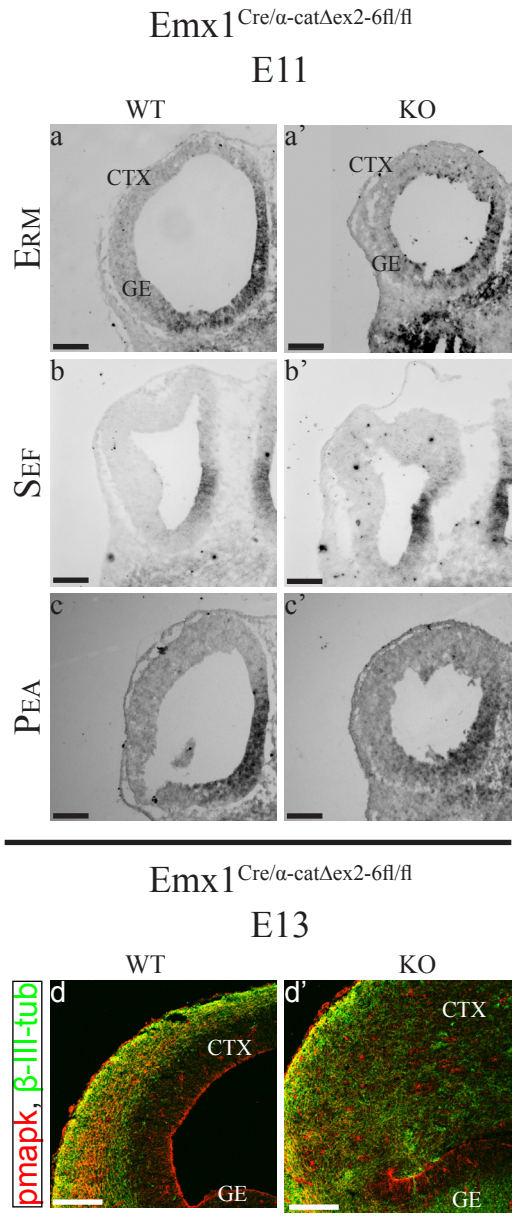


Figure 25
MAP-Kinase signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex
Insitu hybridisation for the MAP-Kinase signalling targets Erm (a,a'), Sef (b,b') and Pea (c,c') revealed that these genes are similarly expressed in WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. Immunostaining for phosphorylated MAP-Kinase at E13 revealed no difference between WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex (d,d').

Abbreviations: CTX: cortex; GE: ganglionic eminences;
Scale bars indicate 100µm;

Figure 26

Notch and canonical Wnt signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex

RT-PCR for Notch signalling targets Hes1 and Hes5 and for the canonical Wnt signalling target Conductin showed that these genes are expressed to similar levels in WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices at E11 (a). However the Topgal reporter revealed increased levels of TCF/LEF transcriptional activity specifically in the rostral cortex (b,b',c,c'). *Insitu* hybridisation for Hes5 at E13 revealed that cortical precursor cells were distributed throughout the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex (e,e') but still expressed Conductin in a medial high to later low gradient as observed in WT (d,d').

Scale bars indicate 100μm;

Figure 26

Notch and c.Wnt signalling in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

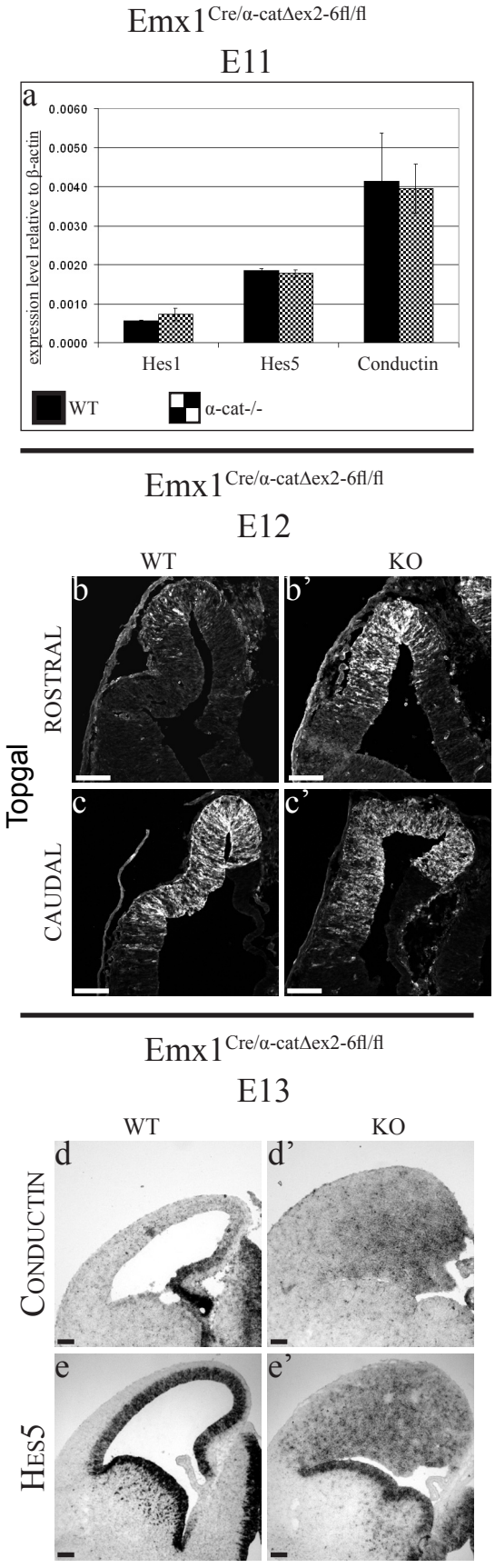


Figure 27

Micro Array analysis and RT-PCR confirmation

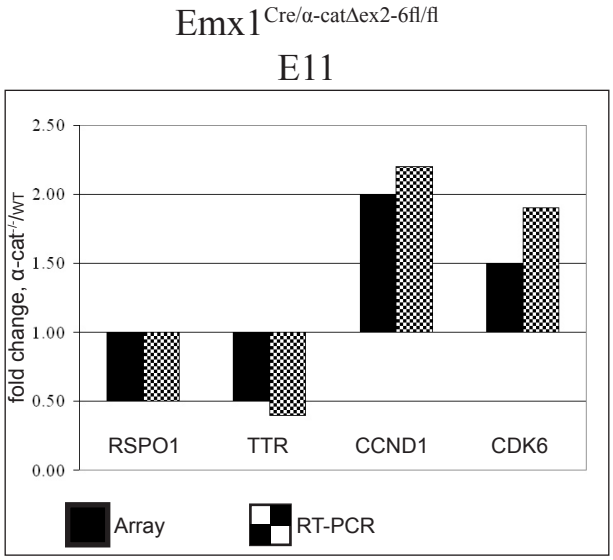


Figure 27

Micro Array analysis and RT-PCR confirmation

Micro Array data from WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex were confirmed by RT-PCR. 4 representative examples are shown. R-spondin (RSPO1), transthyretin (TTR), CyclinD1 (CCND1), Cyclin-dependent kinase 6 (CDK6).

Figure 28

Precursor identity in the α-catenin deficient cortex

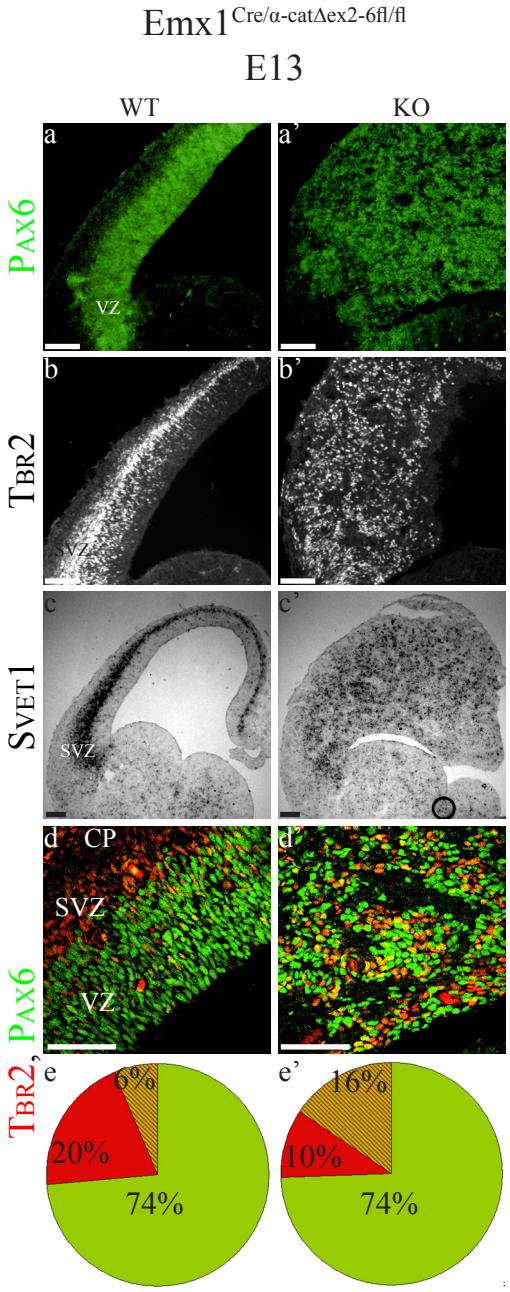


Figure 28

Precursor identity in the α-catenin deficient cortex

Immunostaining for Pax6 revealed the dorsal identity of cortical precursor cells in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ mutant (a,a'). Immunostaining for Tbr2 (b,b') and *insitu* hybridisation for Svet1 (c,c') demonstrate the presence of intermediate progenitor cells in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex. These staining also show the impaired cortical organization as ventricular zone and subventricular zone are not distinguishable in the mutant cortex (a',c'). The populations of radial glial cells and intermediate progenitor cells were identified by double staining for Pax6 and Tbr2, respectively (d,d'). Quantitative analysis of these populations demonstrates that their relative size was not changed, but the population of precursor cells expressing both Pax6 and Tbr2 were doubled in the Emx1$^{Cre/\alpha\text{-}catΔex2fl/fl}$ cortex (e,e').

Abbreviations: vz: ventricular zone, svz: subventricular zone, CP: cortical plate;
Scale bars indicate 100μm;

Figure 29

Neuronal subtype specification in the Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex

Distinct neuronal subtypes were identified by their respective gene expression. Neurons for layer I were immunostained for Reelin (a) and Calretinin (b). Neurons of layer V and VI express Er81 (e) and Tbr1 (c,d) respectively. *Insitu* hybridisation for Cux2 (f,g) depicted neurons of layer II and III. Neurons of all layers were present in the Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex. Most of the neurons in the mutant cortex were not organized in layers but randomly distributed throughout the cortical parenchyma (c'',d'',e'',f'',g''), sometimes in clusters (yellow arrow in e''). At the pial surface few neurons organised into a thin laminar structure (c',d',e',f',g') with neurons born at E14 localised further outside then deep layer neurons immunostained for Tbr1 (d,d'). Neuronal birth dating analyses by BrdU did not reveal abnormalities in neuronal subtype specification as neurons generated at E12 co-localise with Tbr1 (c,c',c'') and those generated at E14 with Cux2 (f,f',f'') in WT and Emx1$^{Cre/\alpha\text{-cat}\Delta ex2fl/fl}$ cortex .

Yellow arrow indicates cluster of neurons;
White arrows indicate cells only positive for BrdU;
Orange arrows indicate cells positive for BrdU and the respective neuronal marker;
Abbreviations: PIA: pial surface; v: ventricular lumen;
Scale bars: 10µm (a,a',b,b'); 100µm (c-g'')

Figure 29

Neuronal subtype specification in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

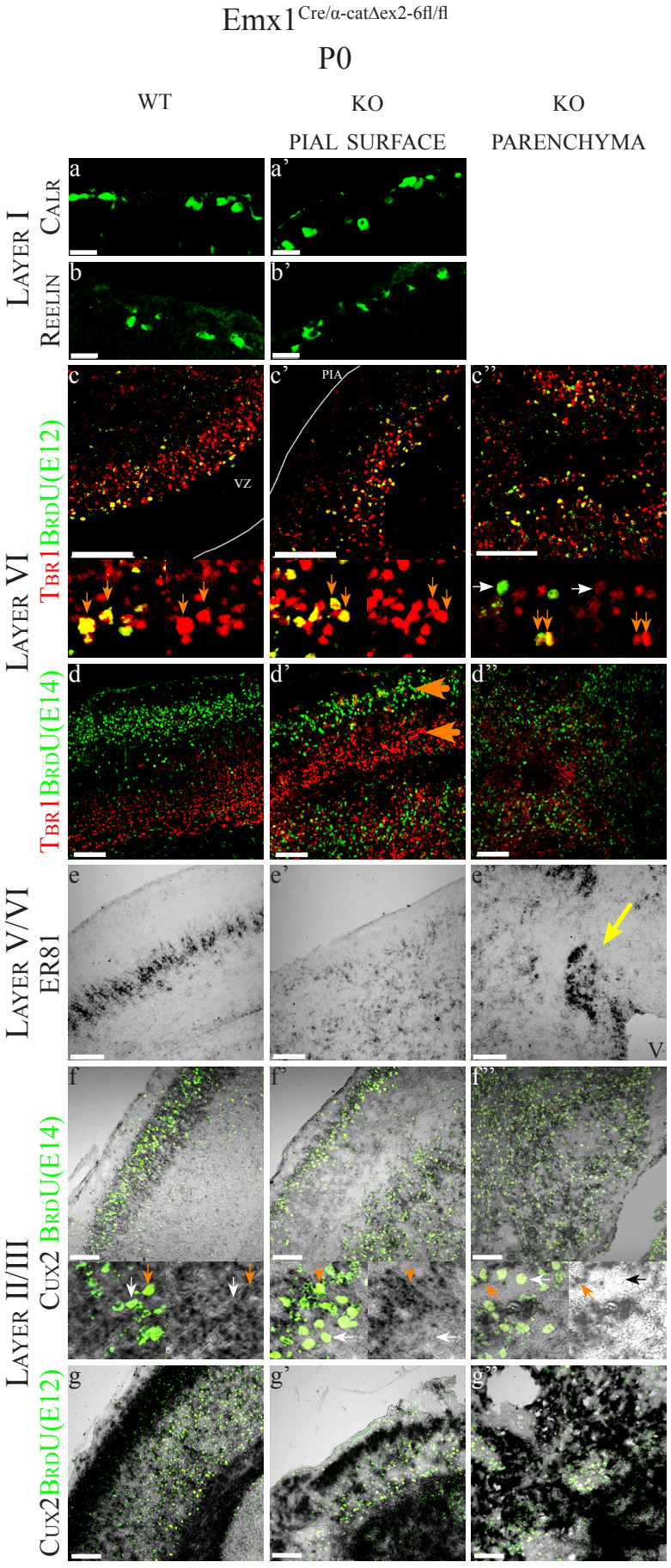


Table : Differentially expressed genes in WT versus Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex

a) MicroArray analysis at E11 revealed 34 genes to be differentially expressed

Id	Gene	Expression WT	Expression α-cat-/-	Carma-Ratio	BH
1451447_at	Cuedc1	62	33	0.3	4.10E-09
1415824_at	Scd2	7	69	2.8	4.64E-09
1456239_at	FGF17*	22	6	0.4	9.36E-07
1422945_a_at	Kif5c	13	35	2.5	9.37E-07
1456112_at	Tpr	33	76	2.5	9.37E-07
1435521_at	Msi2	16	37	2.5	1.15E-06
1428402_at	Zcchc3	101	44	0.4	1.86E-05
1417714_x_at	Hba-a1	2664	781	0.4	4.71E-05
1459737_s_at	Ttr*	679	369	0.4	4.90E-05
1455913_x_at	Ttr*	363	208	0.5	5.47E-05
1417831_at	Smc1l1	26	42	2.2	6.35E-05
1419152_at	2810417H13Rik	11	61	2.2	6.35E-05
1419179_at	Txnl4	40	17	0.5	7.11E-05
1426259_at	Pank3	26	50	2.1	0.00011597
1450035_a_at	Prpf40a	21	46	2.2	0.00011597
1424598_at	Ddx6	29	63	2.1	0.00013187
1453573_at	Hist1h3d	17	35	2.1	0.00016897
1451477_at	1700029I01Rik	5	13	2.1	0.00017865
1418989_at	Ctse	41	17	0.5	0.0002103
1417419_at	Ccnd1	83	168	2.1	0.00021339
1454608_x_at	Ttr*	1075	595	0.5	0.00022481
1457262_at	2610207I05Rik	15	29	2.1	0.00022481
1418562_at	Sf3b1	105	193	2.1	0.00029341
1448877_at	Dlx2	48	88	2.1	0.00037635
1418431_at	Kif5b	9	24	2.0	0.00041219
1421523_at	FGF17*	23	12	0.5	0.00041219
1429433_at	Bat2d	59	130	2.0	0.00041219
1449319_at	Rspo1	226	123	0.5	0.00041219
1425050_at	Isoc1	176	333	2.0	0.00043465
1452360_a_at	Jarid1a	13	26	2.0	0.00043465
1451461_a_at	Aldoc	104	79	0.5	0.00049926
1453172_at	Stch	10	27	2.0	0.00053919
1449145_a_at	Cav1	45	22	0.5	0.00066219
1436343_at	Chd4	81	129	2.0	0.00077532
1455831_at	Fus	88	164	2.0	0.00080322
1452731_x_at	LOC544988	50	89	2.0	0.00080513
1434106_at	Epm2aip1	65	116	2.0	0.00087123

* genes represented with multiple probesets

b) MicroArray analysis at E13 revealed 5 genes to be differentially expressed. Note that Ccnd1 is not upregulated at E13 any longer

Id	Gene	Expression WT	Expression α-cat-/-	Carma-Ratio	BH
1436936_s_at	Xist*	10	1182	54.0	2.5733E-06
1427262_at	Xist*	1	320	70.4	6.7234E-06
1424903_at	Jarid1d	96	3	0.0	4.556E-05
1427263_at	Xist*	1	59	11.2	5.2903E-05
1417210_at	Eif2s3y	119	1	0.0	7.441E-05
1422860_at	Nts	2	48	5.2	0.00018635
1452077_at	Ddx3y*	81	2	0.1	0.00018635
1426439_at	Ddx3y*	41	3	0.1	0.00018635
1426438_at	Ddx3y*	151	1	0.0	0.00021891

* genes represented with multiple probesets

Indeed, the lack of differential expression of Cyclin D1 or any other cell cycle-related gene between WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortices at E13 is consistent with the similar number of progenitors per cortex area in WT and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ littermates at this stage (Tab.I). These data demonstrate that the loss of α-catenin leads to an immediate but transient acceleration of the cell cycle, increasing the pool of progenitors and thus resulting in an increased cortex size.

Discussion

The aim of this work was to address three aspects of the role of β-catenin and α-catenin during the development of the cerebral cortex: first, the time and region specific function of β-catenin, second, which aspects of the function of β-catenin can be attributed to canonical Wnt signalling and which to cellular adhesion and third, how α-catenin influences cortical development.

β-catenin dependent Wnt signalling during cortical neurogenesis

The deletion of β-catenin within the developing cortex revealed that canonical Wnt signalling determines cortical size by controlling the expansion of the progenitor pool. In the absence of β-catenin, cortical precursor cells prematurely leave the cell cycle thereby depleting the precursor pool. This function of canonical Wnt signalling is more prominent during early than late developmental stages and the data presented here support a distinct role for canonical Wnt signalling within the cortical hem. In addition, cell fate analyses revealed that IP cell generation was not affected by the loss of canonical Wnt signalling, while neuronal subtype specification was impaired in the β-catenin deficient cortex. These data therefore show that β-catenin serves different functions during cortical development in a time and region specific manner.

The proliferative role of canonical Wnt signalling is more prominent at the onset of neurogenesis than at midneurogenesis

A distinct caesura in cortical development is the onset of neurogenesis demarcating the change from a purely proliferative to a differentiative behaviour of precursor cells (Götz and Huttner 2005). Prior to neurogenesis, NE cells divide mostly symmetrically to expand the progenitor pool, but change to an asymmetric mode of cell division to generate neurons (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Concurrently with the onset of neurogenesis NE cells become replaced by RG cells that differ in their molecular composition from the former (Götz 2003; Pinto and Götz 2007). We were therefore interested in whether canonical Wnt signalling serves different functions before and after the onset of neurogenesis. To address this question, β-catenin, the main transducer of canonical Wnt signalling, was deleted from floxed alleles (Brault et al. 2001) with Emx1::Cre (Iwasato et al.

2000) before E11 and with hGFAP-Cre (Zhuo et al. 2001) after E13. The two resulting mutants, the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant, displayed distinctly smaller cortices than their WT littermates. However the reduction in cortical size of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant was much more prominent than that of the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant consistent with the decrease of precursor cells by up to 28% in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ but only to 68% in the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. Consistent with previous reports (Backman et al. 2005) deletion of β-catenin after E10 did not influence cortical patterning as dorsal but not ventral TF were expressed in the cortex. Thus defective dorso-ventral patterning did not contribute to the differently pronounced phenotypes of the two β-catenin mutants. Cortical precursor cells in both mutants maintained the expression of Lhx2 but strongly reduced Hes5, Emx1 and Emx2. The expression of Emx2 was demonstrated to be directly controlled by Wnt and BMP responsive enhancer elements (Theil et al. 2002), suggesting that the remaining expression of Emx2 was due to the presence of BMP signalling. Whether the expression of Emx1 is regulated by similar mechanisms is not understood. In general Emx1 and 2 are believed to act in a highly redundant manner as Emx1 mutants show only subtle defects compared to Emx2 mutants (Yoshida et al. 1997). Emx1/2 double-knock out mice in contrast display a cortex, dramatically reduced in size, similar to that of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. Moreover, Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants fail to develop archipallial structures similar to Emx1/2 double-knock out mice (Tole et al. 2000; Shinozaki et al. 2004) and both of the later recombining β-catenin mutants, the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and Nestin$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants were impaired in hippocampus development. In addition to the direct control of Emx2 expression by canonical Wnt signalling, both were reported to closely interact in a positive feedback loop to promote precursor proliferation in the medial cortex (Muzio et al. 2005). This suggests that the impaired proliferation of precursor cells in the β-catenin deficient cortex is at least partially brought about by the reduced expression levels of Emx2. This is further supported by the findings that Emx2 over-expression in cortical precursor cells increases their proliferation by favouring symmetric over asymmetric division (Heins et al. 2001), indicating that with the loss of Emx1 and 2 symmetric divisions are lost and the precursor pool becomes reduced.

In addition to Emx genes, the expression of Hes5 was strongly reduced in the β-catenin deficient cortices. Hes5, a prominent promotor of proliferation, however is known as an expression target of Notch rather than Wnt signalling (Yoon and Gaiano 2005). Together with Hes1, Hes5 was shown to maintain the self-renewing state of precursor cells by counteracting differentiative cell-signals (Ishibashi et al. 1994; Hatakeyama et al. 2004; Mizutani et al.

2007). Interestingly Notch- and Wnt signalling have been reported to interact at various levels of their pathways (Espinosa et al. 2003; Teo et al. 2005; Estrach et al. 2006). For example in skin, the expression of the Notch ligand Jagged was shown to be dependent on β-catenin mediated Wnt signalling, and the absence of β-catenin resulted in decreased activation of Notch (Estrach et al. 2006). Moreover Gsk3β, sequestering β-catenin to degradation in the absence of Wnt signalling, was reported to phosphorylate Notch2, thereby inhibiting Hes gene expression (Espinosa et al. 2003). This is of particular interest, as with the absence of β-catenin the cytoplasmic levels of unoccupied, active Gsk3β should increase and thus be free to phosphorylate other target molecules, as for example Notch2. Thus the absence of β-catenin interferes with the expression of Notch signalling targets suggesting that this contributes to the reduced proliferation observed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and $hGFAP^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants.

Despite this, it should be noted that the expression of Hes5 similar to that of Emx2 is strongly reduced but not entirely lost in the β-catenin deficient precursor cells. This observation is consistent with the notion that Wnt signalling maintains and stabilises rather than induces gene transcription (Parr et al. 1993; Lawrence et al. 2000; Martinez Arias 2003). Loss of function studies in Drosophila and mouse showed that in the absence of Wnt signalling, gene transcription is correctly initiated but subsequently decays (Bejsovec and Martinez Arias 1991; Galceran et al. 2001) and that ectopic activation of Wnt signalling often increases but rarely induces gene transcription (Chenn and Walsh 2002; Megason and McMahon 2002; for review see: Lawrence et al. 2000; Martinez Arias 2003). Also consistent with this notion, was the demonstration that β-catenin acts on gene transcription by recruiting the chromatin remodelling machinery to, already DNA bound, TCF/LEF TF, displacing transcriptional repressors as for example Groucho/TLE (Billin et al. 2000; Barker et al. 2001; Daniels and Weis 2005). With respect to these findings it can be suggested that the reduced precursor pool in the β-catenin deficient cortices is caused by reduced expression levels of various genes that control precursor proliferation. It further implicates a possibility how the effect of Wnt signalling can change with the identity of precursor cells as gene expression patterns differ prior and after the onset of neurogenesis.

β-catenin dependent canonical Wnt signalling promotes cell cycle re-entry and inhibits apoptosis early during neurogenesis

In general, cortical size can become reduced by an increasing cell cycle length, reducing the number of cells generated over time, by an increase in cell cycle exit, depleting the progenitor

pool or by an increase in cell death. Canonical Wnt signalling has been implicated in the latter two, however defects in both processes have not been observed in all examined mutants (Brault et al. 2001; Chenn and Walsh 2002; Machon et al. 2003; Junghans et al. 2005). Closer examination of the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant revealed that deletion of β-catenin in the early developing cortex caused an immediate and extensive withdrawal of progenitor cells from the cell cycle causing premature neuronal differentiation as apparent from the increased number of neurons in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex compared to WT littermates. These results were consistent with data obtained by the conditional deletion of β-catenin in the hindbrain and spinal cord (Zechner et al. 2003) but not with results obtained from β-catenin deletion in the medial cortex by D6-Cre (Machon et al. 2003). However expression of constitutively active β-catenin in cortical precursor cells, driven by a Nestin enhancer element, caused the repeatedly cell cycle re-entry of precursor cells and a dramatic cortical overgrowth (Chenn and Walsh 2002; Chenn and Walsh 2003). Confirming these findings, recent data obtained by in utero electroporation of the Cre-recombinase gene into the β-catenin$^{floxed/floxed}$ cortex strongly suggested that canonical Wnt signalling promotes cell cycle re-entry in a cell autonomous manner (Woodhead et al. 2006). Thus β-catenin dependent Wnt signalling is needed to keep cortical precursor cells proliferating thereby expanding and/or maintaining the progenitor pool.

With the onset of neurogenesis, cortical precursor cells start to divide in an asymmetric, differentiative mode of cell division in addition to the symmetric, proliferative mode that predominates before neurogenesis (Chenn and McConnell 1995; Calegari et al. 2005; Huttner and Kosodo 2005). Prior to neurogenesis proliferative cell divisions serve to expand the pool of precursor cells last but not least to define the final size of the cortex, whereas after the onset of neurogenesis the generation of precursor cells by asymmetric cell divisions maintains but does not expand the pool of precursor cells. Thus it is conceivable that Wnt signalling, promoting cell cycle re-entry promotes the expansion of the precursor pool prior to neurogenesis but assures the maintenance of the precursor pool afterwards. The expansion of the progenitor pool, with 2 precursors generated from one follows an exponential increase whereas divisions maintaining the precursor pool do not. Thus, the effect of sudden cell cycle exit would be expected to be more prominent on symmetric proliferative divisions than on asymmetric differentiative divisions, as observed in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant respectively. A specific promotion of symmetric cell divisions by canonical Wnt signalling is also consistent with the observed increase in symmetric divisions upon the

over-expression of Emx2 (Heins et al. 2001) and with the decline of canonical Wnt signalling during neurogenesis when asymmetric divisions increase.

The examination of apoptosis in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant revealed a 2 fold increase in cell death upon the loss of β-catenin contributing to the reduced precursor pool observed. Similar aberrations in cell death were reported in some (Brault et al. 2001; Zechner et al. 2003), however not in all β-catenin loss-of-function paradigms in the CNS (Chenn and Walsh 2002; Machon et al. 2003; Woodhead et al. 2006). Expression of constitutively active β-catenin rather increased cell death than decreased it, arguing for a secondary defect (Chenn and Walsh 2002) and cell autonomous deletion of β-catenin did not induce cell death, too (Woodhead et al. 2006). Interestingly, in those β-catenin mutants showing increased cell death, β-catenin was deleted at relatively early stages as E10.5 in the fore-, mid- and hindbrain (Brault et al. 2001), E11 in the spinal cord and E8 in the telencephalon (Junghans et al. 2005). Whereas deletion of β-catenin later during development as E13 (Machon et al. 2003; Woodhead et al. 2006) were not reported to induce cell death. In this regard it is noteworthy that the replacement of NE by RG cells, determined by the onset of the expression of the Glutamate transporter GLAST takes place in the mid- and forebrain around E11 and in the spinal cord relatively late about E12 (Pinto and Götz 2007), raising the possibility that β-catenin might contribute to cell survival only in NE but not in RG cells.

If so it wood be expected that apoptosis does not contribute to the reduced progenitor pool of the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant, this however remains to be determined. Independently of the different precursor types that might be affected in the different β-catenin mutants, Junghans et al. (2005) claimed that the apoptotic effect of β-catenin is not attributed to its function in canonical Wnt signalling. This was based on the absence of nuclear β-catenin immunostaining at E9.0, arguing that NE cells lack β-catenin dependent gene transcription. A recent publication by Machon et al. (2007) however used the Bat-gal reporter mouse line to demonstrate the activity of canonical Wnt signalling in the dorsal telencephalon as early as E8.5. Therefore it can not be excluded that canonical Wnt signalling contributes to the observed increase in apoptotic cells in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. Moreover it should be noted that NE cells do not only display AJs to which β-catenin contributes but also β-catenin independent TJs (Anderson et al. 2004; see however :Nunes et al. 2006) and that a definitive proof for the loss of cell adhesion in the developing cortex in the absence of β-catenin has not yet been provided.

The role of canonical Wnt signalling in IP cell generation

Despite the extensive cell cycle exit at E11 some precursor cells were still present in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex at E13. These expressed Ngn2 and Pax6 and further generated neurons throughout development, demonstrating their neurogenic fate. In contrast to NE cells that generate the earliest neurons directly, by asymmetric cell divisions, RG cell give rise to neurons directly or indirectly via the generation of IP cells. Therefore the increased generation of neurons by premature cell cycle exit raised the possibility that the loss of β-catenin induced immediate differentiation of RG cells into neurons, omitting the formation of IP cells. Examination of IP cells by their gene expression revealed that this precursor type was present in both, the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and the hGFAP$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortices, suggesting that the loss of β-catenin did not interfere with the indirect mode of neurogenesis from RG cells. This was further supported by the finding that the proportion of RG cells, expressing Pax6, and the proportion of IP cells, expressing Tbr2 were not changed in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. This is consistent with the notion that the loss of β-catenin specifically inhibits symmetric, proliferative cell division, as the generation of IP cells from RG cells has been observed so far only in asymmetric cell divisions (Huttner and Kosodo 2005). However these data strongly contradict suggestions that have been drawn from β-catenin overexpression experiments(Machon et al. 2007; Wrobel et al. 2007). The absence of Cux2 and Svet1 as well as the reduction of Tbr2 upon the expression of constitutively active β-catenin were claimed that β-catenin functions to maintain RG cell population and to inhibit IP cell generation. However, it should be considered that over-expressing approaches in general increase protein amounts to artificially high levels such that artificial effects can occur. Moreover, cortical precursor cells, expressing constitutively active β-catenin strongly proliferate suggestively in a proliferative, symmetric manner such that they continuously self-renew. This would, by default, prevent the generation of IP cells. Therefore it can not be determined to which extent these highly proliferating cells maintain their neurogenic potential. Indeed, Machon et al 2007 reported decreased levels of Pax6 and Ngn2 expressing precursor cells in the presence of constitutively active β-catenin, suggesting that the ectopic activation of β-catenin interferes with the precursor identity or fate. Recently it has been hypothesised that the acquisition of neurogenic fate and subsequent generation of neurons is correlated with a reduced cell cycle length, supposedly to accumulate molecular neurogenic factors (Calegari et al. 2005). Thus it appears possible that the constitutive activation of β-catenin might suppress neurogenic fate as a secondary consequence of the induced precursor self-renewal. However, the data presented here clearly demonstrate that neurogenic fate and the generation of IP cells can occur

undisturbed in the absence of β-catenin strongly suggesting that canonical Wnt signalling does not actively counteract IP cell generation.

Neuronal subtype specification is disturbed in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex

In addition to their proliferation promoting function, some Wnts have been implicated in neuronal specification. Wnt8b for example, participates in neuronal specification in the hypothalamus, in a manner dependent on the transcription factor Sox3 (Lee et al. 2006). In the telencephalon, canonical Wnt signalling was reported to control the specification of neuronal subtypes of the hippocampus, where it remains highly expressed throughout neurogenesis (Machon et al. 2007). In the developing neocortex, it has only recently been shown that a subpopulation of neurons activates the Topgal reporter construct when electroporated in utero (Woodhead et al. 2006), suggesting that canonical Wnt signalling may play a role in a distinct population of cortical neurons. However, as this was observed in young neurons at midneurogenesis it was not clear whether the Topgal activation was restricted to a distinct subpopulation of neurons or to a distinct developmental phase. Despite these new insights it has not been addressed so far whether canonical Wnt signalling influences neuronal subtype specification in the developing telencephalon.

The early deletion of β-catenin by Emx1::Cre led to an increased cell cycle exit and neuronal differentiation at E11, suggesting a large proportional increase of deep layer neurons, at the expense of upper layer neurons due to the exhaustion of the progenitor pool. Despite this, the remaining precursor cells at E13 expressed Ngn2 and Pax6, genes that are essential to the formation of deep and upper layer neurons respectively (Schuurmans et al. 2004). Consistent with this, neuronal subtypes of all cortical layers were found in the P0 Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. The disorganisation of the β-catenin mutant cortex prevented a quantitative conclusion from the neuronal marker analysis, nevertheless upper layer neurons, identified by the expression of Cux2 and Satb2 appeared with a similar frequency as Tbr1 positive deep layer neurons. As the deletion of β-catenin prior to E11 only excludes early born Cajal-Retzius cells, which have been shown to be generated outside of the cortex (Bielle et al. 2005; Yoshida et al. 2006), these data suggested that β-catenin deficient precursor cells are able to generate neurons of different subtypes and cortical layers. This argues once more for the occurrence of asymmetric cell division in the absence of β-catenin. With respect to the high cell cycle exit upon deletion of β-catenin it appeared possible that parts of these prematurely generated neurons acquired an upper instead of deep layer neuronal identity.

To examine whether some of the upper layer neurons present in the P0 Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex were generated in the early phases of premature neurogenesis BrdU birthdating analyses were performed. In WT, deep layer neurons were mainly generated during early (E11/E12) and upper layer neurons during late (E14-E16) developmental stages, consistent with the established view of cortical genesis (Molnar et al. 2006). In the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex most of the neurons generated at E11 indeed expressed Tbr1 suggesting that cortical precursor cells generate deep layer neurons if they are forced to leave the cell cycle early in development. Upper layer neurons, expressing Cux2, in turn were partially generated at E16, as observed in WT. In contrast to WT however, some of the neurons that were generated at E11 in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex did express markers of upper cortical layers, thus acquired an inappropriate identity. Similarly, neurons born at E16 were found to express deep layer markers that have not been observed in WT. This suggests that the loss of β-catenin interferes with mechanisms of neuronal subtype specification although the majority of neurons were correctly specified. Notably mis-specified deep layer as well as upper layer neurons were found, indicating that the loss of β-catenin does not impair the specification of a particular neuronal subtype. Whether these mis-specifications are directly caused by the absence of canonical Wnt signalling or represent a secondary consequence from the premature neurogenesis at early stages remains to be determined. Nevertheless, the impaired formation of laminae and the consequent inappropriate environment can be excluded as a cause of the observed neuronal mis-specifications, because the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex displays a similar disorganisation but no neuronal mis-specifications.

Different cortical precursor subtypes in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex maintain the generation of cortical neurons

Besides neuronal specification, the BrdU birthdating experiments also clearly revealed that precursor cells persist, although in reduced number, throughout neurogenesis in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. This is surprising, as withdrawal of canonical Wnt signalling is supposed to cause immediate cell cycle exit and also because the Topgal reporter construct is equally activated by all cortical precursor cells (Fig.1u, Woodhead et al. 2006) If so, the continuous generation of neurons throughout development suggests that not all precursor cells immediately leave the cell cycle upon the loss of β-catenin. This would implicate that some precursor cells, although responsive to canonical Wnt signalling do not rely exclusively on Wnt signalling to proliferate. In this regard it is noteworthy that distinct subpopulations of precursor cells have indeed been identified in the cortex (Malatesta et al. 2003; Pinto et al.,

unpublished data; Gal et al. 2006). Interestingly the two RG cell populations that have been identified by different activation of the human promoter of glial fibrillary acidic protein (GFAP) (Pinto et al., unpublished data), differ in their neurogenic potentials as one was found to be mostly neurogenic, generating neurons directly and the other generating neurons predominantly through the generation of IP cells. Although both populations do not show differences in the expression of direct Wnt signalling targets such as Conductin or CyclinD1, the non-neurogenic RG cells expresse higher levels of TCF3, Notch1 and 3 and genes of the BMP, TGF and FGF families as well as cell cycle regulating genes. In addition, the subset of direct neurogenic precursor cells express increased levels of neurogenic related TF such as Pax6 and neuronal differentiation genes like Nr2f2 and Atbf1. Thus, it is conceivable that precursor cells of the indirect neurogenic population do not immediately leave the cell cycle upon the loss of β-catenin as they express high levels of presumably Wnt independent pro-proliferative genes. These cells would thus be able to produce IP cells throughout development, giving rise to late born neurons, acquiring an upper layer fate. The presence of different neuronal subtypes generated even late in cortical development in the $Emx1^{Cre/\beta\text{-}catΔex2\text{-}6fl/fl}$ cortex might thus reflect the so far under estimated heterogeneity of cortical precursor cells.

Canonical Wnt signalling within the cortical hem regulates lateral cortical expansion

Despite the growing number of studies on β-catenin, it remains elusive which Wnt ligands control canonical Wnt signalling in the telencephalon. Only two Wnt genes are expressed throughout the developing cortex, Wnt7a and 7b. Although Wnt7a has been reported to induce neuronal differentiation *in vitro* (Hirabayashi et al. 2004), *in vivo* data or mouse mutant analyses on the cortex are missing. Wnt7b in turn has been shown to be ectopically activated in the Extra-toes mutant supposedly due to neuronal misspecifications (Theil 2005). The Wnt7b knock-out mice however, do not show any obvious defects in corticogenesis (personal observation). In contrast to Wnt7a and b, the majority of Wnt ligands present in the cortex are expressed in the cortical hem, that is believed to act as a signalling centre within the telencephalon (Grove et al. 1998). Signalling centres, such as the isthmic organizer and the anterior neural ridge, have been demonstrated to instruct cell identity, thus controlling neural patterning but also to influence precursor proliferation (Rubenstein et al. 1998; Wurst and Bally-Cuif 2001). Similarly, Wnt ligands, expressed in the cortical hem, have been implicated in dorsal telencephalic patterning as well as in precursor proliferation (Lee et al. 2000; Gunhaga et al. 2003). It therefore seems possible that Wnt ligands expressed in the

cortical hem may also contribute to precursor proliferation in the developing cortex. Interestingly, despite the high number of Wnt ligands expressed in the cortical hem, also including Wnt7a and 7b it is not clear whether canonical Wnt signalling itself occurs within this region. The Wnt signalling target gene Conductin is expressed in the cortical hem, whereas the Topgal reporter construct is not. In contrast the Batgal reporter construct shows weak activity in the cortical hem early but not late in development (Machon et al. 2007). Despite this it should be noted that high levels of the canonical Wnt signalling inhibitor Dkk3 was found to be expressed in the cortical hem (Diep et al. 2004). To functionally address the question whether canonical Wnt signalling within the cortical hem influences cortical development I used the Nestin-Cre line, that does not recombine in the cortical hem to delete β-catenin and to compare the resulting mutant to the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant, that both lacked β-catenin within the cortical hem. The Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant displayed a clearly distinct phenotype compared to the other two β-catenin mutants. Whereas in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ and hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant the cortex was reduced in its tangential expansion, this was not observed in the Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant. Moreover proliferation in the Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ cortex was not reduced to levels comparable with any of the other mutants. This suggested that Wnt signalling in the cortical hem indeed influences cortical expansion, supposedly by regulating proliferation, such that cortical size is significantly reduced when β-catenin is deleted in the entire cortex, including the cortical hem. In addition, ablation of the cortical hem by a diphtheria toxin approach causes a distinct decrease in cortical size but leaves cortical neurogenesis largely intact (Yoshida et al. 2006). Still, it has to be considered that the Nestin$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant also displayed severe malformations within the GE and other regions of the CNS that were not affected by recombination in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ or hGFAP$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutants and that may add to the differences in the phenotypes observed. Moreover, deletion of β-catenin exclusively in the cortical hem, by Wnt3a-Cre, did not cause obvious cortical aberrations (personal observations). However, by recombination with this Cre line β-catenin is lost from the cortical hem at E13 but might be most important at early developmental stages, as suggested by the most prominent phenotype in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant.

The disturbed cortical architecture in the Emx1$^{Cre/\beta\text{-cat}\Delta ex2\text{-}6fl/fl}$ mutant can be allocated to the adhesive function of β-catenin by comparison to α-catenin

Besides the key role in canonical Wnt signalling, β-catenin also participates in the formation of AJs and thereby tightly connects two cellular processes, cell signalling and cell adhesion.

These two processes have been found to be correlated during development, e.g. in epithelial-mesenchymal-transition, as well as in carcinogenesis where decreased cellular adhesion has been shown to permit tumor invasion (Birchmeier and Birchmeier 1995; Gottardi and Gumbiner 2004). β-catenin in AJs, connecting Cadherin molecules to α-catenin, is membrane bound, in contrast to cytoplasic β-catenin that is complexed by APC and Axin in the absence of Wnt signalling and translocated to the nucleus in the presence of Wnt signalling (Bienz 04). Therefore β-catenin can shuttle between a membrane/Cadherin bound state and a cytoplasmic one. Moreover increasing evidence suggests that two distinct pools of β-catenin exist within a cell and that changes in phosphorylation or molecular conformation favours the binding of β-catenin to Cadherin and α-catenin or to TCF/LEF TF and thereby regulates adhesion versus signalling (Gottardi and Gumbiner 2004; Bienz 2005; Brembeck et al. 2006). This dual role of β-catenin and the consequent close correlation of canonical Wnt signalling and cellular adhesion made it difficult to allocate specific aspects of the β-catenin loss of function phenotype distinctly to one of its functions. To address this problem we compared the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant to the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant, in which α-catenin has been deleted by the same Emx1::Cre line as β-catenin. Both mutants showed similar cellular distributions of AJs molecules, F-actin and other proteins that have been shown to localize at AJs. Although these molecules were no longer concentrated at the ventricular surface in the mutant cortices they remained localized in close proximity to each other in the α- or β-catenin deficient cells. Moreover electron microscopy analysis demonstrated that α- or β-catenin deficient cortical cells are indeed able to form cell-cell contacts that resemble AJs. Although both molecules have been reported to be essential to AJs formation and/or maintenance several examples show that this is not always the case. A previous study on the deletion of α-catenin in the skin was shown to abrogate AJs but still allow the localisation of β-catenin and Cadherin to the membrane (Vasioukhin et al. 2001). Similarly, the deletion of α-catenin by a gene trap approach, leading to lethality prior to implantation, still allows the co-localisation of Cadherin and β-catenin at the sites of cell-cell attachment and both molecules can be co-immuno-precipitated (Torres et al. 1997). Moreover, C.elegans humpback (Hmp1, α-catenin homolog) mutants show retraction of actin bundles from the AJs but do not lack AJs themselves (Costa et al. 1998). Similar to α-catenin, reports on β-catenin are inconsistent in regard to the effect of deletion of β-catenin on adhesion. β-catenin knock-out mice, for example, do not develop further than the blastocyst stage displaying cellular detaching supposedly due to impaired adhesion (Haegel et al. 1995). Independently generated β-catenin knock-out mice however develop slightly further and do not display adhesive defects a

phenotype that has been accredited to plakoglobin, substituting for β-catenin (Huelsken et al. 2000). Similarly, plakoglobin was suggested, but not clearly demonstrated, to replace β-catenin upon deletion in the skin (Huelsken et al. 2001). Here we show by electron microscopy that neither β-catenin nor α-catenin is required for the formation of cell-cell adhesion contacts between cortical precursor cells. Despite this however β-catenin and α-catenin deficient cell-cell contacts were not sufficient to maintain radial glial morphology, likely due to an impaired connection between the AJs and the cytoskeleton.

α-catenin was shown to connect the AJs to the cytoskeleton in a highly dynamic manner (Drees et al. 2005; Yamada et al. 2005) and was also reported to interact with several actin remodelling molecules (Verma et al. 2003; Scott et al. 2006). Arp2/3, for example, mediating actin branching is inhibited by α-catenin promoting the formation of actin bundles at the sites of cell-cell contacts (Weis and Nelson 2006). This mechanism might provide an explanation for how the loss of α-catenin weakens the actin cytoskeleton by preventing the formation of actin bundles near the plasma membrane. β-catenin in turn usually links α-catenin to the AJs, thus it is conceivable that the absence of β-catenin prevents a strong interaction between the cell-cell contacts and the cytoskeleton.

RG cells in both mutants, lost their longitudinal shape and acquired a round but multipolar morphology instead, and both mutants showed impaired cortical architecture. As neurons are known to migrate along the radial fibres of RG cells to form the cortical layers it is conceivable that the retraction of these fibres prevents the formation of distinct cortical layers and instead causes the rather random distribution of precursor cells and neurons in the mutant cortices. Similar laminar defects were observed in the mouse retina when β-catenin was deleted after the onset of neurogenesis and thus in the absence of canonical Wnt signalling, further arguing that the disorganisation of the β-catenin deficient cortex is due to the adhesive function of β-catenin (Fu et al. 2006). In addition to their physical adhesive functions, AJs have been found to concentrate several molecules that are involved in cellular polarity. The maintenance of cell-cell contacts in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant therefore suggested the possibility that polarity molecules might still localize to the remaining cell-cell contacts preventing their random distribution throughout the mutant cells. This was indeed the case for Par3 that participates in the Par complex and is thought to localize it to the cell membrane. Moreover prominin, concentrated at the apical patch of cortical precursor cells, was distinctly localized in α- and β-catenin deficient cells. Thus, although the loss of α- or β-catenin impairs the radial morphology of cortical precursor cells, localisation of several polarity molecules is maintained to a certain degree. This contrasts with loss of function

mutants in which the deletion of other apical molecules (e.g. Cdc42, aPKCλ, Lgl, Dlg5, or Numb) results in the maintenance of remnants of AJs but cellular polarity being adversely affected (Klezovitch et al. 2004; Cappello et al. 2006; Imai et al. 2006; Nechiporuk et al. 2007; Rasin et al. 2007). The notion that aspects of cellular polarity, as for example the Par complex, were maintained in the absence of α-catenin was furthermore supported by the finding that the protein levels of Par3, aPKC, Cdc42 and Par6 remained unchanged in the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant compared to WT. The maintenance of the Par complex might be of importance for the finding that IP cells are formed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex and that the proportions of RG and IP cells are maintained. This is especially pertinent as the loss of Cdc42, participating in the Par complex, has been shown to cause the generation of IP cells at the expense of Pax6 positive precursor cells (Cappello et al. 2006) and negative interference with molecules of the Par complex induces premature neurogenesis (Cappello et al. 2006; Imai et al. 2006; Costa et al. 2007).

α-catenin in progenitor proliferation, fate and neuronal specification

The loss of α-catenin causes only minor changes in global gene transcription

Comparison of the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ and $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant cortices strongly suggests that the aberrations in RG morphology, observed in both mutants reflect the shared function of α- and β-catenin in cellular adhesion and thus do not result from impaired canonical Wnt signalling in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. Consistent with the common function in cellular adhesion but not in canonical Wnt signalling, the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant did not display a reduced cortical size as observed in the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant. In contrast, α-catenin deficient precursor cells over-proliferated, substantially increasing cortical size. Besides the complex molecular functions of α-catenin at the junction of adhesion and cytoskeleton (Kobielak and Fuchs 2004; Scott et al. 2006; Weis and Nelson 2006) α-catenin has been reported to affect signalling pathways such as the Wnt signalling (Kioussi et al. 2002; Merdek et al. 2004; Hwang et al. 2005), the MapKinase signalling (Vasioukhin et al. 2001) and the SHH signalling pathway (Lien et al. 2006b). However so far it has not been possible to allocate a specific function in any of these pathways to α-catenin.

In contrast to previous studies, recombination by Emx1::Cre deleted α-catenin in a discrete region of the CNS, namely the cerebral cortex. The data obtained from our micro array confirmed results from *insitu* hybridisation, demonstrating the absence of ectopic activation of SHH or FGF signalling targets and revealed a two fold increase in the expression of the cell

cycle promoting gene CyclinD1. The over-proliferation together with the increased expression of CyclinD1 in the concurrent absence of activated SHH targets in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant strongly contradict the suggestion made by Lien et al, that α-catenin would control cell proliferation by inhibiting SHH target activation in a cell-autonomous manner. However it should be noted that the approach used by Lien et al (2005) to delete α-catenin, affected the entire CNS due to the Nestin-Cre line used (Lien et al. 2006b) including the ventral telencephalon containing a prominent source of SHH (Ericson et al. 1995). Thus it is conceivable that the impaired tissue coherence, reported in this mutant might have allowed the diffusion of SHH molecules into the ventricular lumen. As it has been shown that cortical precursor cells are able, in principle, to respond to SHH molecules (Dahmane et al. 2001) it appears likely that the observed expression of SHH target genes in the cortex of this mutant were indeed caused by SHH signalling.

Besides the absence of SHH target activation, our micro array analysis revealed that only a few genes were differentially regulated upon the deletion of α-catenin. As the micro array was performed on cortical tissue collected at E11 and at E13 it is unlikely that we missed the activation of any genes, especially as the over-proliferation phenotype manifested within the first 24 hours of α-catenin absence. In the group of down-regulated genes, several were included that are known to have functions in the nervous system, including Transthretin (TTR) which is expressed in the Choroid plexus (Dickson et al. 1985) and FGF17 that functions in cortical arealisation (Cholfin and Rubenstein 2007). However, the differentially regulated genes did not point to the deregulation of a distinct cell signalling pathway neither did they represent a homogeneous group sharing a common biological function. Interestingly, micro array data obtained from cortical tissue collected at E13 showed that the differences in gene expression observed at E11 were not present at this stage any more. Strikingly, those genes differentially expressed at E13 were mostly localized on sex chromosomes, e.g. Jarid1d, Eif2s3y and Ddx3y localised at the Y-chromosome and Xist localised at the X-Chromosome, suggesting that the most prominent differences between the WT and Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutants examined had been their sex.

Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ precursor cells transiently accelerate the cell cycle due to increased expression of CyclinD1

The data obtained from the micro arrays, showing an up-regulation of CyclinD1 at E11 but not at E13, were confirmed by the functional analysis of the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant cortex. This revealed that cortical precursor cells shortly after α-catenin deletion reduce their cell

cycle length, leading to over-proliferation with a concurrent reduction in the proportion of generated neurons. At E13 however the proportion of mitotic cells was no longer increased in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant compared to WT, suggesting that the α-catenin deficient precursor cells had overcome the phase of accelerated cell cycle. Despite the regained normal cell cycle length, the cortical size of the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant increases considerably until P0. This demonstrates how a relative moderate expansion in the precursor pool - the number of mitotic cells was increased to 115% in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex at E11 - can enlarge the cerebral cortex by several times. A normalization of proliferation as in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant has not been observed in the Nestin-Cre driven α-catenin mutant (Lien et al. 2006a), most likely due to a continuous activation of SHH signalling, resulting in a persistingly increased proliferation.

By analysing target gene expression, we could exclude that the increased expression of CyclinD1 observed in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex was caused by ectopic activation of SHH or FGF signalling. Furthermore, the global gene transcription analyses in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant cortex showed only minor changes, consistent with the fact that α-catenin so far has not been reported to serve direct functions in gene transcription. Instead, the best characterized function of α-catenin is to connect the AJs to the cytoskeleton, thereby interacting with a variety of different molecules that could be affected in their activity by the absence of α-catenin. The most prominent molecule in this regard is β-catenin that has been reported to bind to α-catenin in the cytoplasm, thereby promoting its localization to the membrane (Gottardi and Gumbiner 2004). Indeed, examination of the Topgal reporter revealed an increased TCF/LEF transcriptional activity in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. This is consistent with experiments done in chondrocytes that showed increased activation of the Topflash reporter construct upon the knock down of α-catenin by siRNA (Hwang et al. 2005). However, the increased Topgal activity was only observed in the rostral parts of the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant cortex, where it is not active in WT, as canonical Wnt signalling activity follows a caudal high to rostral low gradient. The ectopic activation of TCF/LEF transcriptional activity particularly at rostral levels coincides with the increase in cortical expansion that was also most prominent at rostral levels. At caudal levels in contrast, where Wnt signalling is high, TCF/LEF transcriptional activity can eventually not be further increased. Moreover β-catenin is actively degraded by a highly efficient machinery that therefore can balance the pool of β-catenin, which might provide an mechanism to explain the transience of the proliferation defect, observed in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant. Despite this, Topgal reporter acticity was only increased at rostral levels whereas precursor proliferation

and cortical size were increased in the entire cortex, arguing that increased TCF/LEF transcriptional activity may add to, but not be exclusively responsible for the accelerated cell cycle. In this regard it should be noted that the expression of Conductin that has been reported to be directly regulated by β-catenin/TCF signalling (Jho et al. 2002; Lustig et al. 2002) was not changed in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. Conductin is believed to function in a negative feed back loop to limit duration or intensity of canonical Wnt signalling (Jho et al. 2002). However, its expression does not entirely coincide with the Topgal activation, e.g. in the cortical hem, suggesting that it is regulated by additional factors that are not provided in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant. Taken together, these data suggest that the increased TCF-LEF transcriptional activity in the rostral cortex represents a part of the mechanism by which loss of α-catenin accelerates the cell cycle of cortical precursor cells.

In general α-catenin interacts with two cellular components, the AJs and the cytoskeleton, that have been both shown to interact with a broad variety of molecules that in turn can participate in cell cycle regulation. Many internal cell signalling molecules localize in proximity to AJs, like GTPases of the Rho and Rac family as well as Cdc42 together with their guanine-exchange factors as e.g. IQGAP (Perez-Moreno and Fuchs 2006; Pugacheva et al. 2006). Similarly, members of the planar cell polarity pathway that can be induced by Wnt ligands have been found to localize at the apico-lateral membranes of epithelial cells (Veeman et al. 2003; Cadigan and Liu 2006; Seifert and Mlodzik 2007). On the other side α-catenin interacts with the actin cytoskeleton and several actin remodelling molecules (Kobielak and Fuchs 2004). The actin cytoskeleton itself is of great importance to cell division especially to mitosis and cytokinesis and has been reported to influence cell cycle parameters (Nelson 2003; van Opstal et al. 2005; Walker et al. 2005). Thus, it is conceivable that the observed over-proliferation upon the loss of α-catenin is not only caused by the activation of β-catenin dependent gene transcription but rather may be the result of a general imbalance in the distribution of membrane and/or cytoplasmic proteins, that together cause the accelerated cell cycle, but as soon as the cell re-establishes the intracellular balance of these molecules, the cell cycle returns to normal levels.

RG morphology is essential to cortical architecture but not to neuronal subtype specification

Despite the increased and decreased proliferation in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant respectively and the impaired RG cell morphology observed, RG cells as well as IP cells were generated in both mutants. However, whereas the populations of RG and

IP cells remained unchanged in their relative proportion to each other in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutant, in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex, the population of IP cells that express Tbr2 and Pax6 was slightly increased. Both genes, Pax6 and Tbr2 have been shown to be expressed in a temporal sequence with Pax6 preceding Tbr2 (Englund et al. 2005). Taking into consideration that α-catenin deficient precursor cells accelerated their cell cycle it appears possible that newly generated precursor cells did not have enough time to down-regulate Pax6 but started to express Tbr2. As Tbr2 is expressed in neurons prior to E12, this hypothesis can not be tested at E11 when the cell cycle acceleration is most prominent. However, as the Tbr2 positive population in general represents only 25% of the cortical precursor cells, this difference in gene expression of IP cells was restricted to 10% of the entire cortical precursor population.

In addition to the generation of IP cells in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ mutant, neurogenesis occurred throughout development and neurons of different subtypes were specified. As cortical architecture was broadly disturbed in both mutants, neurons of different subtypes largely intermingled or appeared in clusters instead of residing in laminae. A small population of neurons however, was arranged in a lamina, formed directly beneath the pial surface. This thin band became established during development and consisted of late as well as early born neurons, the former settling above the latter. In the WT cortex, neurons cross already existing laminae to settle above them, to establish new cortical layers (Molnar et al. 2006). They do so by migrating either along the radial processes of RG cells or by somal translocation (Casanova and Trippe 2006). In the latter case neurons possess a basal process connected to the basement membrane and pull themselves upwards. As neuronal migration along RG processes is prevented in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex because α-catenin deficient RG cells retract their processes, migration by somal translocation might explain how the thin band of cortical layers could be formed in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. However, so far it has been believed that neurons undergo somal translocation after they inherite the basal process from their RG mother cell (Nadarajah and Parnavelas 2002). This is unlikely to account for those neurons, of the thin layered band in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex, that were born at E14 when RG cells had already retracted their processes.

Besides this minor population, most neurons in the Emx1$^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex were scattered or clustered similar to those in the Emx1$^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ cortex. This observation is consistent with other mutants showing disorganisation of cortical architecture due to impaired RG cell morphology. The deletion of N-Cadherin caused impaired cellular adhesion and subsequent loss of radial processes (Kadowaki et al. 2007) similar to the defects observed upon

interference with the KIF3 molecular motor, that regulates the post-Golgi transport of N-Cadherin (Teng et al. 2005). Although cortical organisation was impaired already at early developmental stages in these mutants, detailed analysis of neuronal subtypes and birth-dating has not been performed. Our findings now demonstrate that cortical architecture is not essential to neuronal subtype specification as neurons of all cortical layers were present in the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ cortex. Furthermore the birth-dating analyses revealed the generation of different neuronal subsets in their appropriate sequence and thus confirmed that cortical precursor cells are endowed with certain intrinsic cues that allow them to sequentially generate neurons (Qian et al. 1998; Qian et al. 2000; Shen et al. 2006) and do not rely on their environmental niche. This has so far only been demonstrated *in vitro* for individually cultured precursor cells (Qian et al. 1998; Qian et al. 2000; Shen et al. 2006) but not tested *in vivo*. Other experiments however have clearly shown that precursor cells generate neuronal subtypes in response to their environment, as young precursor cells generate upper layer neurons when transplanted into a developmentally older cortex (McConnell 1988; McConnell and Kaznowski 1991; Frantz and McConnell 1996). This suggests, that cortical precursor cells do not require environmentally signals to sequentially generate neurons, but they are able to respond to their environment, presumably to act in concert with other precursor cells. Although the lack of cortical architecture does not interfere with cortical precursor identity and fate, both the $Emx1^{Cre/\alpha\text{-}cat\Delta ex2fl/fl}$ and the $Emx1^{Cre/\beta\text{-}cat\Delta ex2\text{-}6fl/fl}$ mutants demonstrate the importance of RG cell morphology for cortical layer formation.

The comparative analysis of α-catenin and β-catenin mutants was used to address the function of Wnt signalling and the importance of morphological integrity of cortical precursor cells, thereby uncoupling the two important functions of RG cells in cortical development: to act as cortical precursor cells and as the cortical scaffold. This revealed novel insights into the distinct role of canonical Wnt signalling and cortical architecture in cerebral cortical development. Canonical Wnt signalling controls the re-entry of cortical precursor cells into the cell cycle supposedly by promoting symmetric proliferative divisions and is thus especially important during early corticogenesis. While the absence of β-catenin impaired neuronal subtype specification but not IP cell fate, the loss of α-catenin caused subtle changes in the latter and increased proliferation but did not disturb neuronal specification. Thus both molecules interfere with precursor proliferation, at least partially by reciprocally affecting Wnt signalling but were clearly not essential to AJs formation/maintenance as shown by

electron microscopy. Moreover all mutants investigated in this work demonstrated that intact RG cell morphology is a prerequisite for the establishment of cortical architecture.

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Präsentationen

2005: Time and region specific function of β-catenin during cortical development in mouse; (Cortical development – neural stem cells to neural circuits, May 12-15, Santorini; Greece)

2006: The role of adherens junctions and their signalling complexes in the maintenance of neural stem cells; (Embryonic and somatic stem cells – regenerative systems for cell and tissue repair, September 24-27, Dresden; Germany)

2007: Neural precursor fate – the role of apical signals, (The 5th international student seminar, Kyoto university 21st century COE program; February 27 – March2, 2007, Kyoto, Japan)

The role of adherens junctions in neural precursor fate and polarity, (Tht 2nd CDB-GSF meeting; July 9-10, Munich, Germany)

Der strebsame Mensch muß nichts fürchten, wenig glauben und alles hoffen.

Johann Nestroy

Mein Dank gilt allen die es mir ermöglicht haben nichts fürchten zu müssen, aber alles hoffen zu dürfen.