A local Wnt-3a signal is required for development of the mammalian hippocampus

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SUMMARY

The mechanisms that regulate patterning and growth of the developing cerebral cortex remain unclear. Suggesting a role for Wnt signaling in these processes, multiple Wnt genes are expressed in selective patterns in the embryonic cortex. We have examined the role of Wnt-3a signaling at the caudomedial margin of the developing cerebral cortex, the site of hippocampal development. We show that Wnt-3a acts locally to regulate the expansion of the caudomedial cortex, from which the hippocampus develops. In mice lacking Wnt-3a, caudomedial cortical progenitor cells appear to be specified normally, but then underproliferate. By mid-gestation, the hippocampus is missing or represented by tiny populations of residual hippocampal cells. Thus, Wnt-3a signaling is crucial for the normal growth of the hippocampus. We suggest that the coordination of growth with patterning may be a general role for Wnts during vertebrate development.

Key words: Wnt, Hippocampus, Pattern formation, Proliferation, Cortical patterning

INTRODUCTION

The embryonic dorsal telencephalon of vertebrates contains the cerebral cortex, which is the seat of higher cognitive functions in mammals. The cerebral cortex is subdivided into many histologically and functionally distinct regions. The broadest subdivisions are between the six-layered isocortex (neocortex) and the non-six-layered allocortices, which include the archicortex and the paleocortex, as well as transitional cortices (Zilles and Wree, 1995). The neocortex and the allocortex are further subdivided into unique areas based on cytoarchitectural and connectional specialization.

The hippocampus is an archicortical structure located at the caudomedial edge of the neocortex (Amaral and Witter, 1995). In the adult, the longitudinal axis of the hippocampus forms a ‘C’-shape. From its rostral pole, located just dorsal and posterior to the septal nuclei, the hippocampus curves over and behind the diencephalon to the incipient temporal lobe, its caudal pole. The transverse axis of the hippocampus is divided into distinct fields. From proximal to distal they are the dentate gyrus (DG) and the CA3 and CA1 fields of Ammon’s horn (the CA fields are often referred to as the hippocampus proper). Lateral to the hippocampus is the subiculum and the pre/parasubiculum, transitional cortices that lie between the hippocampus and the adjacent neocortex.

While little is known about the mechanisms by which the cerebral cortex is divided into allocortex and neocortex, a number of studies are beginning to provide clues about how specific areas are specified within the neocortex. A number of lines of evidence reveal an instructive role for thalamic inputs in specifying the functional properties of neocortical areas (reviewed in O’Leary et al., 1994). For example, if fetal visual cortex is transplanted into neonatal somatosensory cortex, the transplanted tissue gives rise to a somatosensory cortex with many of its normal properties (Schlaggar and O’Leary, 1991). A corollary of these studies is that positional information must be present in the cortical primordia prior to the ingrowth of thalamic axons in order to guide them to their appropriate target areas. The existence of pathways regulating the establishment of such positional information can be inferred directly from a number of studies examining the mechanisms that specify the differential expression of genes in different cortical areas (Barbe and Levitt, 1991; Arimatsu et al., 1992; Ferri and Levitt, 1993; Barth and Stanfield, 1994; Cohen-Tannoudji et al., 1994; Bulfone et al., 1995; Ebrahim-Gaillard and Roger, 1996; Levitt et al., 1997). While these studies all point to the existence of positional information within the early cortical primordia, the molecular mechanisms responsible for establishing this information remain obscure.

Developmentally the hippocampus arises at the caudomedial edge of the continuous dorsal telencephalic neuroepithelium adjacent to the telencephalic roof (Stanfield and Cowan,
A strip of cortical progenitor cells along this edge, the cortical hem (or hem), expresses many members of the Bone Morphogenetic Protein (BMP) and Wnt families of inductive signaling factors (Furuta et al., 1997; Grove et al., 1998). A number of studies have revealed roles for BMP and Wnt signaling in the regulation of dorsal patterning at more caudal regions of the vertebrate neuraxis (Dickinson et al., 1994; Liem et al., 1995, 1997; Arkell and Beddington, 1997; Ikeya et al., 1997; Lee et al., 1998). Thus the cortical hem is a candidate source of information regulating the induction and/or patterning of hippocampal development at the caudomedial margin of the continuous cerebral cortical neuroepithelium.

Wnt-3a expression marks the cortical hem from 9.75 dpc (Roelink and Nusse, 1991; Parr et al., 1993; Grove et al., 1998) and is the only Wnt gene expressed exclusively in the cortical hem at this time, suggesting a role for Wnt-3a signaling in the induction and/or patterning of the hippocampus. In Wnt-3a mutants, medial hippocampal fields are absent and lateral hippocampal fields are severely reduced. We show that Wnt-3a acts at 10.5 dpc to regulate the proliferative expansion of caudomedial cortical progenitor cells. The loss of this ‘hippocampal’ progenitor pool leads to a failure of normal hippocampal development in Wnt-3a mutants.

**MATERIALS AND METHODS**

**Embryo collection, histology and RNA in situ analysis**

The Wnt-3a mouse line was maintained on an outbred Swiss Webster background and genotyping was performed as described (Takada et al., 1994; Yoshikawa et al., 1997). Embryos from heterozygous intercrosses were dissected into PBS and fixed in either 4% paraformaldehyde (for RNA in situ analysis and TUNEL) or in Bouin’s fixative (for histological analysis and bromodeoxyuridine (BrdU) detection). For analysis of tissue sections, embryos were either sectioned at 5-7 μm on a conventional microtome or 40 μm on a freezing microtome. Sections for general histology were stained with haematoxylin and eosin. All analyses were performed using previously published protocols: whole-mount RNA in situ hybridization according to Parr et al. (1993) with modifications described in Knecht et al. (1995), section in situ hybridization with 35S-labeled probes according to Wilkinson et al. (1987), section in situ hybridization with digoxigenin (DIG)-labeled probes was performed according to Tole et al. (1997), and TUNEL analysis according to Gavielli et al. (1992).

**BrdU detection and cell counting**

Pregnant females were injected intraperitoneally with 50 μg BrdU/g body weight and killed 30 minutes later. The uterine horns were immediately removed into ice-cold PBS. Embryos were dissected and processed as described above (note that exclusively 5 μm

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**Fig. 1.** Expression of Wnt genes during early cerebral cortical development. (A-L) Whole-mount in situ hybridizations using digoxigenin (DIG)-labeled probes. (M-R) In situ hybridizations to tissue sections using 35S-labeled probes. At 8.5 dpc (A-C), prior to cephalic neural tube closure, Wnt-7b and Wnt-8b are expressed in similar domains along the dorsocaudal edge of the telencephalon (B,C) with the expression of Wnt-7b extending further rostrally than Wnt-8b (black arrowheads mark the telencephalon/diencephalon boundary). By 9.5 dpc (D-I), after cephalic neural tube closure, the domain of Wnt-8b expression (E,H) comes to lie at the caudomedial edge of the dorsal telencephalon. The expression of Wnt-7b (F,I) is broader than Wnt-8b, and includes much of the dorsal telencephalon. At both 8.5 dpc (A) and 9.5 dpc (D,G), Wnt-3a is expressed in the dorsal CNS with an anterior limit at the prosomere 2/prosomere 3 (p2/p3) boundary in the anterior diencephalon. Wnt-3a expression initiates in the dorsal telencephalon at 9.75 dpc and at 10.5 dpc (J-L) Wnt-3a (J) and Wnt-8b (K) are expressed in nested domains at the caudomedial edge of the dorsal telencephalon. Wnt-8b expression extends further rostrocaudally and laterally than the domain of Wnt-3a expression, which is limited to the presumptive cortical hem. (M-R) At 12.5 dpc, six Wnt genes are expressed in the dorsal cerebral cortex. Wnt-2b, Wnt-3a, Wnt-5a and Wnt-7b are expressed adjacent to the choroid plexus (CP) in the cortical hem (white arrowheads in M-P). Wnt-8b is expressed in a much broader domain along the medial wall of the telencephalic vesicle. A second site of Wnt-8b expression is the eminentia thalami, ventral to the CP. In all cases, Wnt gene expression is extremely low or undetectable in the CP. Wnt-7a is expressed broadly in the cortical neuroepithelium, but is excluded from the cortical hem. Between 10.5 dpc and 12.5 dpc Wnt-7a expression in the cortical neuroepithelium turns off and a second phase of Wnt-7b expression is initiated in the cortical mantle layer (P). Abbreviations: CP, choroid plexus.
Fig. 2. Histological analysis of cerebral cortical development in 18.5 dpc Wnt-3a mutants. Sections were stained with haematoxylin and eosin. (A) In coronal sections through the rostral hippocampus of an 18.5 dpc wild-type brain, the hippocampus is located beneath the neocortex and bulges into the lateral ventricle from its medial edge. (B) In contrast, there is no morphological sign of hippocampal development in Wnt-3a mutant brains. At higher magnification, a dense pyramidal cell layer indicative of the CA fields and the subiculum is easily identifiable in the wild type (C), but not the Wnt-3a mutant (D). The most medial cortical tissue present in the mutant appears to be the cingulate and retrosplenial fields of the neocortex (D). (E,F) In horizontal sections, the subiculum, CA fields and dentate gyrus (DG) are identifiable in wild-type brains but absent in Wnt-3a mutants. Note that the pre/parasubiculum and entorhinal cortex lie between the caudal hippocampus and the neocortex in the wild type (E). In Wnt-3a mutants, the entorhinal cortex and a reduced pre/parasubiculum appears to be the most medial cortical structure present. (G) In coronal sections, immediately rostral to the rostral end of the hippocampus, the hippocampal commissure crosses the midline, ventral to the corpus callosum and dorsal to the anterior commissure. In Wnt-3a mutants, the hippocampal commissure is entirely absent and the axons that would normally cross the midline to form the corpus callosum fail to cross and instead form ectopic Probst bundles medially on either side of the midline. (H) In contrast the anterior commissure forms normally. Abbreviations: AC, anterior commissure; CA1, hippocampal field CA1; CA3, hippocampal field CA3; CC, corpus callosum; CP, choroid plexus; DG, dentate gyrus; Cing/Rsp, cingulate/retrosplenial neocortical fields; Ent, Entorhinal cortex; F, Fimbria; HC, hippocampal commissure; Ncx, neocortex; Pb, Probst bundle; PS, preparasubiculum complex; S, subiculum; Th, thalamus.

Analysis of Wnt gene expression during dorsal telencephalic development

A number of Wnt genes are expressed during development of the dorsal telencephalon in various vertebrate species (Roelink and Nusse, 1991; Parr et al., 1993; Cui et al., 1995; Hollyday et al., 1995; Kelly et al., 1995; Grove et al., 1998; Lako et al., 1998). As a first step to addressing the role of Wnt signaling during dorsal telencephalic development, we performed a comprehensive analysis of the expression of Wnt family members throughout the period of embryonic dorsal telencephalic development in the mouse, 8.0-18.5 days postcoitum (dpc). Of 16 Wnt genes analyzed, six were found to be expressed during this process; Wnt-2b, Wnt-3a, Wnt-5a, Wnt-7a, Wnt-7b and Wnt-8b (Grove et al., 1998) and this study). Expression of Wnt-7b and Wnt-8b first appears in the dorsal telencephalon at the 5- and 3-somite stages, respectively (S. M. K. L. and A. P. M., unpublished data). By the 12-somite stage, Wnt-7b and Wnt-8b are expressed in similar patterns, in the dorsoposterior region of the future dorsal telencephalon (Fig. 1B,C). Note that the domain of Wnt-7b expression extends further anteriorly than that of Wnt-8b, while the expression of both Wnts broadens to extend more medially (future ventrally) at the posterior border of the telencephalon with the diencephalon (black arrowheads in Fig. 1B,C). At this time, Wnt-3a is expressed dorsally in the spinal cord, hindbrain, midbrain and diencephalon to an anterior boundary at the presumptive prosomere 2 (p2) / prosomere 3 (p3) boundary (black arrowhead in Fig. 1A).

At 9.5 dpc, Wnt-7b and Wnt-8b are expressed in nested domains in the dorsal telencephalon. Wnt-8b is expressed in a wedge of cells along the caudomedial edge of the dorsal telencephalic vesicle (Fig. 1E,H) while Wnt-7b is broadly expressed throughout the entire dorsal telencephalon (Fig. 1F,I). Expression of Wnt-3a still maintains an anterior limit at the p2/p3 boundary (Fig. 1D,G). Wnt-3a expression initiates in
the dorsal telencephalon at 9.75 dpc (Roelink and Nusse, 1991; Parr et al., 1993). By 10.5 dpc, the expression domains of all three Wnt genes are nested with respect to the caudomedial edge of the dorsal telencephalon. Wnt-3a is expressed in a narrow band of cells immediately abutting the caudomedial edge (Fig. 1J). This domain of Wnt-3a expression is nested within a broader domain of Wnt-8b expression (Fig. 1K). Note that the domain of Wnt-8b expression extends further rostrocaudally and mediolaterally than the domain of Wnt-3a expression (compare Fig. 1J,K). Wnt-7b is expressed throughout the entire cortical neuroepithelium by this time (Fig. 1L). These expression domains implicate Wnt signaling in the regulation of growth and patterning of the dorsal telencephalic neuroepithelium prior to neural differentiation, which begins at 10 dpc in the dorsal telencephalon.

At 12.5 dpc, six Wnt genes display restricted expression in the dorsal telencephalon. Expression of Wnt-2b, Wnt-3a and Wnt-5a is restricted to a longitudinal band of cells at the junction of the choroid plexus (CP) and the cortical neuroepithelium, the cortical hem (Grove et al., 1998) (Fig. 1M-O). Interestingly, the longitudinal axis of the cortical hem predicts the location of hippocampal development, at the caudomedial edge of the neocortex, Wnt-7a, in contrast, is expressed throughout the dorsal and lateral cerebral cortex but is specifically excluded from medial regions (Grove et al., 1998) (Fig. 1R). Wnt-8b is expressed in a broad graded fashion along the caudomedial wall of the dorsal cerebral cortex in a domain that includes the cortical hem (Fig. 1Q). Finally, Wnt-7b expression in the ventricular zone is restricted to the cortical hem by this time (Fig. 1P). Interestingly, as Wnt-7b expression is turned off by ventricular zone cells expression is initiated in the postmitotic cells which are forming the cortical mantle layer (preplate in neocortical regions) (Fig. 1P; S. M. K. L. and A. P. M., unpublished data), suggesting a role for Wnt signaling in the future path of this axon tract.

In summary, expression of multiple Wnt genes marks the dorsocaudal edge of the dorsal telencephalon from 8.5 dpc. Following closure of the rostral neuroneuroepithelium, this domain is identifiable as the caudomedial edge of the dorsal telencephalon, the cortical hem, and predicts the future site of hippocampal development at the margin of the cerebral cortex.

Hippocampal development requires Wnt-3a

The expression of multiple Wnt genes in the cortical hem, adjacent to the site of hippocampal development, suggests the involvement of Wnt signaling in the regulation of hippocampal development. As Wnt-3a is the earliest Wnt gene to be expressed exclusively in the cortical hem, between 9.75 and 11.5 dpc, we began to address this issue by analyzing cerebral cortical development in Wnt-3a mutant mice (Takada et al., 1994). Histological sections through 18.5 dpc Wnt-3a mutant brains were examined to assess hippocampal development in the absence of Wnt-3a. In coronal sections through the rostral hippocampal formation of wild-type brains the pyramidal cell layer of the subiculum and the CA1 and CA3 fields, as well as the dentate granular cells which are beginning to form the DG and the fimbria/ fornix are all easily identifiable (Fig. 2A,C). In contrast, none of these structures are identifiable in Wnt-3a mutants (Fig. 2B,D). The most medial cortical structure in the mutant brain contains a broad cortical plate, characteristic of neocortex and abuts the choroid plexus of the lateral ventricle (Fig. 2D). Note that the choroid plexus of the lateral ventricles always forms in Wnt-3a mutants but may be reduced in size (Fig. 2A,B,E,F). In horizontal sections through the caudal end of the hippocampal formation of a control brain, the hippocampus and adjacent pre/parasubiculum and entorhinal cortex are identifiable (Fig. 2E). (Note that the pre/parasubiculum and entorhinal cortex are only present at more caudal levels of the hippocampal longitudinal axis). In Wnt-3a mutant brains, similarly to more rostral levels, no hippocampal structures are identifiable (Fig. 2F). The adjacent multilayered entorhinal cortex, however, appears to be present and possibly some subicular fields (Fig. 2F).

Immediately adjacent to the rostral pole of the hippocampus, the fimbria becomes the fornix and the commissural component of the fornix forms the hippocampal commissure (Amaral and Witter, 1995) (Fig. 2G). The corpus callosum crosses the midline immediately dorsal to the hippocampal commissure (Fig. 2G). In Wnt-3a mutants, both the hippocampal commissure and the corpus callosum fail to form (Fig. 2H), while a small ectopic bundle of axons (a Probst bundle) is present between the retrosplenial cortex and the choroid plexus, which is likely formed by misrouted colossal axons (Fig. 2D). This suggests either that Wnt-3a signaling is directly required for corpus callosum formation or that the hippocampus or hippocampal commissure provide a growth substratum and/or directional cues required for colossal axons to cross the midline.

The morphological absence of hippocampal development in Wnt-3a mutants could result from a failure to generate hippocampal precursor cells or from the failure of hippocampal precursors to develop into a hippocampus once they are generated. To address whether hippocampal field-specific cell types are formed in Wnt-3a mutants, and to define the defects in the medial cortex with a cellular resolution, we used a panel of region-specific molecular markers. Brains from mutant and control mice were first analyzed at 15.5 dpc, the earliest age at which hippocampal subfields can be readily identified with molecular probes. In controls at this stage embryonic CA3 is marked by KA1 expression (Fig. 3A) (Wisden and Seeburg, 1993; Bahn et al., 1994; Tole et al., 1997) while CA1 and the DG can be identified by patches of Steel expression (Fig. 3C) (Motro et al., 1991; Tole et al., 1997). However, in the brains of 15.5 dpc Wnt-3a mutants, no CA1, CA3 or DG cells can be identified by either marker (Fig. 3B,D). In both control and mutant brains, a dense band of neocortical cells expresses Steel (Fig. 3C,D). Interestingly, in the mutant, this band of neocortical Steel expression extends almost to the edge of the cortex, a further indication that little or no hippocampus is present.

Brains were next analyzed at 18.5 dpc. At this age, more hippocampal molecular markers are expressed and each hippocampal subfield is larger. For both reasons, any residual hippocampal cells in the mutant brain should be easier to identify. In control brains, CA3 is identifiable by KA1 expression, CA1 and the DG express Steel, and the subiculum expresses BIG-1 (Fig. 3E,G,I) (Motro et al., 1991; Wisden and...
Seeburg, 1993; Bahn et al., 1994; Yoshihari et al., 1994; Tole et al., 1997). In some mutant brains, a small population of CA3 cells is now detectable by KA1 expression (Fig. 3F), but no DG cells are ever detectable by Steel expression (Fig. 3H). The subiculum and CA1 remain undetectable at most rostromedial levels through the mutant brain (Fig. 3HJ). However, very caudally, at a level at which CA1 and the subiculum normally appear at their largest in coronal sections, small patches of Steel-positive CA1 and BigI-positive subicular cells can be identified (data not shown). Finally, a small pre/parasubicular complex is evident in the mutant by expression of Steel and NT3 (data not shown). By contrast with the gross shrinkage or absence of all hippocampal fields in the mutant, NT3 expression, which marks the adjacent cingulate and retrosplenial cortices (Freidman et al., 1991; S. T. and E. G., unpublished observations), reveals that these neocortical areas are strikingly close to normal in size (compare Fig. 3K and L), given the overall slight shrinkage of the mutant cerebral cortex.

In summary, both morphological and molecular marker analysis reveal a severe reduction in hippocampal development in Wnt-3a mutants. Interestingly, the severity of the defects are graded along both the mediolateral and longitudinal axes of the archicortex. At the medial edge of the hippocampal formation the DG is entirely absent at all longitudinal levels. In contrast, CA3, CA1 and the subiculum are absent rostrally and severely reduced caudally. At the lateral edge of the hippocampal formation the pre/parasubicular complex, which is only present caudally in wild-type mice, is reduced but present in Wnt-3a mutants. This severe reduction in hippocampal development suggests that an early defect within the caudomedial cortical neuroepithelium from which the hippocampus develops may underlie the mutant phenotype.

**The caudomedial cerebral cortical wall is shortened in Wnt-3a mutants**

In 12.5 dpc wild-type brains, the caudomedial wall of the cerebral cortex forms a characteristic S-shaped curve, which presages the future morphology of the hippocampal formation (Fig. 4A). In contrast, there is a severe shortening of the caudomedial wall archicortical neuroepithelium in Wnt-3a mutant brains (Fig. 4B). As a result, the choroid plexus (CP), which forms adjacent to the archicortical neuroepithelium and loops into the lateral ventricle at this time, is pulled taut in the mutant (Fig. 4A,B).

The shortening of the medial telencephalic wall in Wnt-3a mutants suggests either that specific medial neural progenitor cell populations are entirely missing or alternatively that these populations are present but significantly reduced in size. In order to distinguish between these alternatives, we analyzed the expression of a panel of genes whose expression marks specific domains of the medial wall neuroepithelium and the choroid plexus at 12.5 dpc. The cortical hem is marked by the expression of Wnt-3a (Fig. 4C) (Roelink and Nusse, 1991; Grove et al., 1998) and the absence of Bf-1 expression (Furuta et al., 1997) (Fig. 4E). Msx-1 expression marks the choroid plexus (Furuta et al., 1997) (Fig. 4G), which forms ventral to the cortical hem at the medial border of the cerebral cortical neuroepithelium, and also the very most ventral portion of the cortical hem itself. Finally, Wnt-8b is expressed in a broad domain of the medial wall neuroepithelium (Fig. 4I). Thus the expression of these markers unambiguously defines three progenitor cell populations in the medial wall: Msx-1 marks the choroid plexus, the cortical hem is Wnt-3a-positive and Bf-1-negative, and the domain of graded Wnt-8b expression marks a broad domain of caudomedial wall neuroepithelium. In Wnt-3a mutants, the choroid plexus expresses Msx-1 despite its abnormal morphogenesis (Fig. 4H). Within the neuroepithelium, complementary Wnt-3a and Bf-1 expression marks the cortical hem (Fig. 4D,F), which is somewhat reduced in size relative to wild type (compare with Fig. 4C and E). (Note that Wnt-3a expression can be detected in Wnt-3a+/−embryos using a probe that hybridizes to sequences outside of the disrupted region.) Thus the cortical hem and choroid plexus are formed and maintained in the absence of Wnt-3a signaling. In contrast, the graded medial wall expression of Wnt-8b is absent and only the cortical hem expression remains (Fig. 4I).

Thus a large domain of caudomedial cortical neural progenitor cells, abutting the cortical hem, is absent by 12.5 dpc. This implies that Wnt-3a acts prior to this stage and is required for either the specification or maintenance/expansion of this progenitor cell pool.

To determine the exact onset of the loss of caudomedial cortical progenitors in Wnt-3a mutants, we performed a histological analysis of dorsal telencephalic development between 9.75 dpc (the time of initial Wnt-3a expression in the dorsal telencephalon) and 11.5 dpc. Up to 10.5 dpc, development of the mutant dorsal telencephalon is indistinguishable from wild type (data not shown). However, by 10.75 dpc, there is a dramatic shortening of the caudomedial cortical neuroepithelium in Wnt-3a mutants relative to controls (compare Fig. 5B,F with A,E).

In summary, the caudomedial cortical neuroepithelium of Wnt-3a mutants fails to undergo a proper morphological elongation and this results in a significant reduction in the size of the caudomedial cortical wall. While the choroid plexus and the cortical hem neuroepithelial populations are specified and maintained, a large population of caudomedial neural progenitors, which normally expresses Wnt-8b, is missing by 12.5 dpc, implying a requirement for Wnt-3a signaling to either specify or maintain/expand this neuroepithelial cell population.

**Wnt-3a is required to maintain neural progenitor cell proliferation in the caudomedial cortical neuroepithelium**

Wnt-3a could act to maintain/expand neural progenitor cells via at least three distinct mechanisms: as a mitogenic factor required to stimulate proliferation, as a survival factor required to inhibit apoptosis or as an antendifferentiation factor required to maintain neural progenitors in a proliferative, undifferentiated state. We addressed each of these possibilities in turn.

To ask whether Wnt-3a is required for neural progenitor cell proliferation, we directly assayed cell proliferation in the telencephalic neuroepithelium at 10.75 dpc, at the onset of the caudomedial neuroepithelial defect, by monitoring the incorporation of bromodeoxyuridine (BrdU) into S-phase nuclei. To quantify the proportion of neuroepithelial cells that were proliferating, we first defined the 200 neuroepithelial cell nuclei that were closest to the roof (this is approximately the population of cells that lies between the bars in Fig. 5E,F and corresponds approximately to the domain of Wnt-3a expression at this time). Note that, in the mutant, this

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The population is within the morphologically abnormal region of the caudomedial neuroepithelium. We next determined the number of nuclei, out of these 200, that had incorporated BrdU (the mitotic index). Calculations were based on six wild-type and six Wnt-3a mutant embryos, scoring two sections per embryo. We observed a 26% reduction in the proportion of labeled cells in the medial (morphologically abnormal) wall of the dorsal telencephalon of Wnt-3a mutants as compared to wild-type littermates (Student’s $t$-test, $P<0.0001$) (Fig. 5C-F). In contrast, there was no significant difference in the proportion of labeled cells when a lateral control region (approximately the region between the bars in Fig. 5G,H) was compared between Wnt-3a mutant and wild-type littermates (Student’s $t$-test, $P=0.41$). Thus Wnt-3a plays a critical role in maintaining neural progenitor cell proliferation in the caudomedial dorsal telencephalon.

We next asked whether Wnt-3a is required for neural progenitor cell survival. While we routinely observed characteristically high levels of cell death in the telencephalic
roof of both control and mutant embryos, we never observed any significant ectopic telencephalic cell death in the medial neuroepithelium of mutants (data not shown). Therefore, ectopic cell death does not contribute to the loss of neuroepithelial cells in Wnt-3a mutants.

A third mechanism by which the loss of Wnt-3a signaling could lead to a loss of neural progenitor cells is if Wnt-3a is normally required to maintain neural progenitors in an undifferentiated state. In this scenario, the absence of Wnt-3a would lead to the premature differentiation of neural progenitors into neurons, which would have the effect of

**Fig. 5.** Bromodeoxyuridine (BrdU) incorporation in Wnt-3a mutant brains at 10.5 dpc. Sections were stained with haematoxylin and eosin (A,B) or processed for immunohistochemical detection of BrdU and counterstained with haematoxylin (C,D). Nuclei that have incorporated BrdU are brown while those that have not are light purple. C and D are near adjacent sections to those in A and B. E-H are high-power views of the regions indicated in C and D. In coronal sections through the telencephalon of wild-type embryos (A) the roof (arrowheads in A and B) is flanked by the elongating caudomedial cortical walls of the paired telencephalic vesicles. In Wnt-3a mutants (B), the roof epithelium is morphologically abnormal and the caudomedial cortical walls are shortened (B). Note that in the morphologically abnormal medial cortical neuroepithelium of Wnt-3a mutants there is a marked reduction in the proportion of nuclei which have incorporated BrdU relative to the similar region in controls (E,F). In contrast, the proportion of nuclei incorporating BrdU in lateral cortical regions is indistinguishable between Wnt-3a mutants and controls (G,H).

**Fig. 6.** Analysis of neural differentiation in Wnt-3a mutants. 10.5 dpc wild-type (A,C) or Wnt-3a mutant (B,D) embryos were stained as whole mounts using probes against delta-1 (dl-1) (A,B) or class III B-tubulin (B-tub) (C,D). In all panels dorsal views of the cortex are shown and rostral is oriented down. dl-1 is expressed transiently by differentiating neurons as they leave the cell cycle, and expression appears in a punctate pattern throughout the cortex of both wild-type and Wnt-3a mutant embryos. B-tub marks differentiated neurons and is expressed in the cortical mantle zone of both wild-type and Wnt-3a mutant embryos. For both markers there is a slight reduction in the level of expression at the caudomedial margin of the cortex in Wnt-3a mutants (arrows in B,D).

**Fig. 7.** Analysis of regionally expressed markers in the dorsal telencephalon of 10.5-10.75 dpc Wnt-3a mutants. (A,C,E,G) Wild-type or Wnt-3a mutant (B,D,F,H) embryos at 10.5 dpc (A-D) or 10.75 dpc (E-H) were stained as whole mounts. In all panels, dorsal views of the cortex are shown and rostral is oriented down. Wnt-3a is expressed in the presumptive cortical hem at the caudomedial edge of the cerebral cortical neuroepithelium (A) in a domain which is nested within a broader domain of Wnt-8b expression (C). Wnt-8b expression extends further rostrocaudally and mediolaterally than Wnt-3a expression (compare C with A). In Wnt-3a mutants, Wnt-3a is expressed normally in the presumptive cortical hem (B). In contrast, there is a significant reduction in the lateral extent of the Wnt-8b expression domain (D), such that much of the expression beyond the cortical hem is lost (compare D with C). Bmp4 is expressed along the medial and dorsocaudal margin of the cortical neuroepithelium in both wild-type (E) and Wnt-3a mutant (F) embryos. Finally, Bf-1 is expressed throughout the cortical neuroepithelium but is specifically excluded from a caudomedial domain which approximately corresponds to the domain of Wnt-3a expression in both wild-type (G) and Wnt-3a mutant (H) embryos.
prematurely depleting the progenitor cell pool and result in too few neurons being generated. In order to investigate this possibility, we analyzed the expression of the neurogenic gene delta-1 (dl-1), which is transiently expressed by neural precursor cells soon after they withdraw from the cell cycle (Bettencourt et al., 1995), and the neural-specific class III B-tubulin (B-tub), a marker of differentiating neurons (Burgoyne et al., 1988; Grove et al., 1998). Both genes are expressed in a reticulated pattern throughout the mantle zone of the dorsal telencephalon at 10.75 dpc (Fig. 6A,C). In Wnt-3a mutants, the cortical expression of both dl-1 and B-tub is normal or perhaps slightly reduced at the caudomedial edge (arrows in Fig. 6B,D), where the hippocampal primordium lies. This result argues against a requirement for Wnt-3a to suppress neuronal differentiation.

In summary, while we failed to observe an increase in cell death or any premature neuronal differentiation in Wnt-3a mutants, we did observe a significant reduction in neural progenitor cell proliferation specifically in the region of Wnt-3a signaling. Thus, the loss of neural progenitor cells in the caudomedial cortical neuroepithelium of Wnt-3a mutants is at least partially the result of a decreased level of proliferation in these cells.

Wnt-3a is required for the expansion of the caudomedial cortical wall, the site of the hippocampal primordium

The observed reduction in caudomedial cortical progenitor cell proliferation at 10.75 dpc and the subsequent truncation of the caudomedial neuroepithelium by 11.5 dpc implies that Wnt-3a driven proliferation is necessary for the expansion of a pool of caudomedial progenitor cells, which normally form the caudomedial cortical wall. To investigate this issue, we analyzed the expression of Wnt-3a and Wnt-8b between 10.5 and 10.75 dpc in Wnt-3a mutant and control embryos. Between 10.5 and 10.75 dpc, the nested expression of Wnt-3a and Wnt-8b in the caudomedial cortical neuroepithelium defines two adjacent populations of progenitor cells in controls (Fig. 7A,C). A domain of cells abutting the caudomedial cortical edge expresses both Wnt-3a and Wnt-8b, while more lateral cells express only Wnt-8b. The Wnt-8b-expressing sector thus contains a broad region of caudomedial cortex, which, by its position, is highly likely to include the hippocampal primordium. In Wnt-3a mutants, nested domains of Wnt-3a and Wnt-8b expression are indistinguishable from controls up to 10.5 dpc (data not shown). However, beginning at 10.75 dpc, there is a marked reduction in the lateral extent of the Wnt-3a expression domain (Fig. 7D), while the medial domain of Wnt-3a and Wnt-8b co-expression remains intact (Fig. 7B,D). These observations suggest that, while the cortical hem is maintained in Wnt-3a mutants, the hippocampal primordium is greatly reduced by 10.75 dpc.

BMP-mediated dorsal patterning events do not require Wnt-3a

The Wnts are not the only secreted factors with precise domains of expression within this area of the brain. Beginning at 8.0 dpc multiple BMP-encoding genes are expressed in the dorsal telencephalon in domains that variably include the roof/choroid plexus and the cortical hem (Furuta et al., 1997; Grove et al., 1998). In cultured explants of 10.5 dpc dorsolateral cortex, BMP-coated beads induce ectopic apoptosis and Msx-1 expression, while inhibiting Bf-1 expression, suggesting a role for BMP signaling in the induction of roof/choroid plexus and perhaps cortical hem cell fates and/or the inhibition of dorsolateral cortical cell fates (Furuta et al., 1997). To address the possibility that some of the defects in Wnt-3a mutants are mediated by a reduction in BMP signaling, we assayed Bmp4 and Bf-1 expression in the dorsal telencephalon of 10.75 dpc mutant and control embryos. In both mutant and control embryos, BMP-4 expression is limited to the telencephalic roof and midline head mesenchyme (Fig. 7E,F) and Bf-1 is expressed throughout the cortex but is excluded from a caudomedial domain that includes the roof and the presumptive cortical hem (arrows in Fig. 7G,H). Further, in Wnt-3a mutants cell death is normal and Msx-1 expression in the choroid plexus is unaltered (data not shown and Fig. 4H). These observations indicate that some patterning events that are presumed to be downstream of BMP signaling occur normally in Wnt-3a mutants.

**DISCUSSION**

The patterning of the vertebrate brain occurs through the stepwise subdivision of the neuroepithelium into an orthogonal grid of regional domains, which are the primordia of adult anatomical structures (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Shimamura et al., 1995; Lumsden and Krumlauf, 1996; Rubenstein et al., 1998). Within each regional domain, a specific and highly complex array of neural cell types are generated. The processes of regionalization and cell type specification appear to be mediated by the action of inductive signaling factors (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996; Rubenstein et al., 1998). It is thus the local interaction of these inductive factors with neural progenitor cells that determines the spatial relationship between different anatomical structures and the types of neural cells contained within each structure of the adult brain. An intriguing problem is that of how growth is controlled in coordination with these primary patterning processes in order to generate anatomical structures of the appropriate size made up of the appropriate numbers of each cell type. Our data argue that Wnt-3a is required for the expansion of a caudomedial cortical progenitor pool that generates the hippocampus. We suggest that it is the coordination of Wnt-3a-mediated proliferation with hippocampal cell fate specification that is responsible for the generation of a normal hippocampus.

**A local Wnt-3a signal is required for the expansion of a population of hippocampal progenitor cells in the caudomedial wall of the cerebral cortex**

In Wnt-3a mutants, the initiation of dorsal telencephalic development, as indicated by the establishment of Bmp-4 expression in the roof, and the specification of caudomedial cortical cell fates, as indicated by the establishment of complementary Wnt-3a and Bf-1 expression domains, occurs normally. However, our data indicate that Wnt-3a is required to promote the proliferation of cortical progenitor cells at the caudomedial margin of the cerebral cortical neuroepithelium. In Wnt-3a mutants, this region shows a marked decrease in proliferation, which most likely accounts for the failure in
expansion of the caudomedial wall of the cerebral cortex. Interestingly, the shortened caudomedial wall of mutants maintains a domain of Wnt-3a-expressing cells along its margin but is missing a large domain of rostromedially adjacent progenitors, which normally express Wnt-8b. This implies that the domain of Wnt-3a-expressing cells, which require Wnt-3a for their proliferation at 10.75 dpc, normally grows to generate the caudomedial wall of the cortical neuroepithelium and that over time caudomedial cortical progenitor cells, which initially express Wnt-3a, expand to occupy more rostromedial positions and turn off Wnt-3a transcription.

The initiation of dorsal telencephalic Wnt-3a expression at 9.75 dpc and the subsequent initiation of hippocampal neuronal birth at 10.5 dpc (Angevine, 1965; Stanfield and Cowan, 1988) suggests that Wnt-3a signaling is associated with the production of hippocampal neurons. The presence of small residual clusters of CA1, CA3 and subicular cells at the caudomedial margin of the Wnt-3a mutant cortex suggests that hippocampal cells are specified in the mutant but that the failure of Wnt-3a driven hippocampal progenitor cell expansion results in a depletion of the reduced progenitor cell pool once hippocampal neuronal birth begins. Thus, it may be the coordination of the production of a locally acting mitogenic signal, Wnt-3a, with the primary patterning processes responsible for inducing hippocampal development and specifying hippocampal cell types that determines the size of the hippocampus.

The local regulation of proliferation may be a general role for Wnt signaling during vertebrate CNS development. Studies of dorsal hindbrain and spinal cord development have implicated BMP and Wnt signaling in the regulation of dorsal CNS patterning (Dickinson et al., 1994; Liem et al., 1995, 1997; Arkell and Beddington, 1997; Ikeya et al., 1997; Lee et al., 1998). This process is initiated by BMP signaling from the non-neural ectoderm, which induces neural crest formation from early dorsolateral neural progenitors concomitantly with the induction of roof plate (Liem et al., 1995). BMP signaling from the roof plate subsequently acts to specify the differentiation of dorsal neuronal cell types from dorsolateral neural progenitors (Liem et al., 1997). Wnt-1 and Wnt-3a are expressed in the dorsal CNS from 8.0 dpc (Wilkinson et al., 1987; Roelink and Nusse, 1991; Hollyday et al., 1995) and their expression can be ectopically induced in intermediate neural explants following recombination with non-neural ectoderm (Dickenson et al., 1995), suggesting that Wnt-1/3a signaling acts downstream of BMP signaling in the dorsal CNS. Ectopic expression and loss-of-function studies have identified a critical role for Wnt-1/3a signaling in the dorsal hindbrain and spinal cord. Ectopic expression of Wnt-1 throughout the DV axis of the spinal cord leads to overgrowth of neural progenitors but does not affect the expression of molecular markers of DV pattern (Dickinson et al., 1994). In complementary studies, the analysis of mouse embryos mutant for both Wnt-1 and Wnt-3a reveals a severe reduction in dorsolateral neural progenitors and a subsequent failure to generate many neural crest derivatives (Ikeya et al., 1997). Together with our findings, these studies suggest that the expansion of dorsal neural progenitor cells by Wnt-1/3a signaling is a common role for Wnt signaling during vertebrate CNS development.

In Drosophila, the local expression of wingless (wg) (also known as DWnt-1) in the wing pouch is required for local proliferation in the wing hinge (Neumann and Cohen, 1996), while local wg expression in the Malpighian tubule anlage is required for proper elongation of the Malpighian tubules (Skaer and Martinez Arias, 1992). Conversely, the ectopic expression of wg in either of these tissues leads to local overgrowth but does not affect primary patterning (Skaer and Martinez Arias, 1992; Neumann and Cohen, 1996). Thus the local regulation of proliferation may be a general role for Wnt signaling during pattern formation.

Implications for the mechanism of hippocampal field specification

Cell lineage studies have indicated that the field identity of hippocampal neurons is not determined by cell lineage, arguing that hippocampal fields are not generated by restricted pools of progenitor cells that are permanently specified or committed to produce neurons of a single field (Grove et al., 1992; Walsh and Cepko, 1992). Clues as to the timing of CA field specification come from the observation that molecular markers of CA1 and CA3 (SCIP and KA1, respectively) are first expressed in the presumptive CA1 and CA3 (Pca1 and Pca3) fields at 15.5 dpc (Tole et al., 1997). In slice cultures of Pca1 or Pca3 isolated at 17.5 dpc, later aspects of mature CA field identity develop autonomously (Tole et al., 1997). The origin of these field-specifying signals is suggested by the observation that the expression of SCIP in Pca1 and KA1 in Pca3 initiates in presumptive CA pyramidal cells at the subicular (lateral) and dentate (medial) poles of the presumptive CA region, respectively. Over a period of 3-4 days, fronts of SCIP and KA1 expression gradually sweep across the hippocampal primordia until the expression of these two markers complementarily defines the Pca1 and Pca3 fields in their entirety (Tole et al., 1997). Together, these observations imply that signals from the medial and lateral poles of the hippocampus are responsible for specifying CA field identity and that these signals act on presumptive CA neurons prior to 15.5 dpc.

The expression of multiple Wnt genes in the cortical hem (Grove et al., 1998 and this study), adjacent to the medial edge of the developing hippocampus, together with the known ability of Wnt signals to specify cell fate choices acting as both local inducers and long-range gradient morphogens in a number of developmental contexts (eg.: Vincent and Lawrence, 1994; Zecca et al., 1996; Neumann and Cohen, 1997; Dorskey et al., 1998; and see Cadigan and Nusse, 1997 and Moon et al., 1997 for recent reviews), suggests the possibility that cortical hem-derived Wnt signaling is involved in the specification of medial hippocampal fields. In Wnt-3a mutants, the hippocampal progenitor cell pool is dramatically reduced in size and this results in a severe reduction in hippocampal development. However, small populations of cells expressing markers of CA1, CA3 and the subiculum can still be specified. This is consistent with the idea that these fields are normally specified by other signals originating from the subicular and dentate poles of the hippocampus (see above) (Tole et al., 1997), and suggests that these signals are still active in Wnt-3a mutants. The dentate gyrus normally develops adjacent to the cortical hem domain of Wnt-3a expression and we observed a complete absence of dentate gyrus development in Wnt-3a mutants. This is consistent with the possibility that Wnt-3a

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signaling normally specifies dentate gyrus cell fates. A definitive interpretation of this result is, however, difficult in the context of the dramatic reduction in overall hippocampal development and the fact that the timing of specification of dentate gyrus cell fates is unknown. Experiments in which Wnt-β-a is ectopically expressed may help to clarify this issue. An additional implication of this observation is that the dentate gyrus is not the source of signals responsible for specifying the identity of adjacent CA3 cells. Finally, expression of Wnt-2b, Wnt-5a, Wnt-7b and Wnt-8b is maintained in the cortical hem of Wnt-α-a mutants. Thus, it is possible that Wnt family members have redundant roles in the specification of hippocampal fields.

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