Title page

Mice lacking *Wnt2b* are viable and display a postnatal olfactory bulb phenotype.

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Abbreviations: A-P, anterior-posterior; PS, primitive streak; OpV, optic vesicle; OtV, otic vesicle; OfB, olfactory bulb; Hp, hippocampus; SVZ, subventricular zone

Abstract

Wnts are secreted glycoproteins that play important roles in embryonic development. Wnt2b is transiently expressed in the primitive streak (PS) during gastrulation and in several organs during organogenesis. To determine the biological function of *Wnt2b* during mouse development, we established a conditional null allele of *Wnt2b*. Mice lacking *Wnt2b* were viable, fertile, and displayed a normal life span, however, the olfactory bulb in adult *Wnt2b* mutant mice was significantly reduced in length. Our results suggest that *Wnt2b* primarily plays a supportive role in gastrulation and organogenesis, functioning redundantly with canonical Wnts, such as Wnt2, in numerous tissues.

Key words: Wnt; embryogenesis; brain; redundancy; olfactory bulb

Wnt proteins encode a large family of cysteine-rich secreted glycoproteins that play established roles in embryonic development and tumorigenesis [16, 17, 25]. Nineteen Wnt genes have been identified in murine and human genomes. These Wnts were initially functionally classified into two groups based on their activity in in vitro transformation assays and 2º-body axis induction assays in Xenopus embryos. Generally speaking, Wnt1-class Wnts (canonical Wnts), which include Wnt1, 3a, 8a and 8b, transform cells and induce the formation of an additional body axis when overexpressed in frog embryos, while Wnt5a class Wnts, such as Wnt4, 5a and 11, do not [5].

Wnt2b was first identified by degenerate RT-PCR and designated *Wnt13* [28]. Wnt2b binds the CRD domain of Frizzled4 and 5 to stabilize cytosolic βcatenin, suggesting that Wnt2b is a canonical Wnt [11]. *Wnt2b* is transiently expressed in the mesoderm of the PS at mid-gastrulation stages only (E7-7.75) [25, 28] and is later expressed in numerous embryonic tissues including the optic vesicle (OpV)/retina, otic vesicle (OtV), kidney, lung, and limb bud, as well as the adult ovary [3, 7, 11, 14, 20, 28]. *Wnt2b* is also expressed in the developing mouse brain at several developmental stages. It's expressed in the dorsal midline of the telencephalon and the mesencephalon at E8.5-9.5, cortical hem at E11.5-17.5, hippocampal fimbria at E15.5-17.5 and pineal body. Based on these observations, *Wnt2b* has been proposed to play a role in the patterning of the cerebral cortex however a requisite role for *Wnt2b* in these processes has not been rigorously tested.

A targeting vector for the *Wnt2b* locus was constructed as follows. A 5.9 kb genomic *Xba*I fragment for long arm 1 (LA1) and a 2.1 kb genomic *Xba*I/*Eco*RI fragment for LA2, which contains exon 2 and 3 for deletion, were inserted into *Sal*I and *Sma*I sites of pML8 vector, respectively. A 1.2 kb genomic *Eco*RI/*Kpn*I fragment for short arm (SA) was inserted into the *Sac*II site of pK-11 vector, which contains a loxP-FRT-*PGKNeo*-FRT cassette for positive selection. An *Xho*I/*Xba*I fragment of LA1-loxP-LA2 isolated from pML8-LA1-LA2 was subcloned into *Xho*I/*Xba*I sites of p*PGKtk*, which contains a *PGKtk* cassette for negative selection. A *Kpn*I/*Sac*I fragment of loxP-FRT-*PGKNeo*-FRT-SA isolated from pK-11-SA was inserted into the *Xba*I site of p*PGKtk*-LA1-loxP-LA2. The targeting vector was linearized with *Not*I for transfection into ES cells. *Not*I-linearized *Wnt2b* conditional targeting vector DNA (80 μg) was electroporated into 8 x 10^7 of W9.5 ES cells (x2 pulses of 320 V/125 μF), and the cells were cultured on a layer of mitotically inactivated feeder cells carrying genomic *PGKNeo* gene. ES cell colonies that were resistant to 300 μg/ml of G418 and 1 μM gancyclovir were independently isolated. These clones of ES cells were screened by Southern blot analysis of *Kpn*I-digested genomic DNA (15 μg) using a 370 bp 5' probe or *Xba*I-digested genomic DNA using a 540 bp 3' probe (Fig. 1A and B). *Wnt2b*$^{WT/flox}$ ES clones were injected into albino C57BL/6N blastocysts to generate chimeras. Chimeric male mice that displayed a high contribution of agouti coat color were bred with C57BL/6N female mice to obtain a germ line-transmitted *Wnt2b*^{flox} allele. *Wnt2b*$^{WT/flox}$ mice were then bred with βactin-Cre transgenic mice to delete exon 2

and 3 to generate the $Wnt2b^{\Delta}$ allele. $Wnt2b^{WT/flox}$ and $Wnt2b^{WT/\Delta}$ mice were intercrossed to generate $Wnt2b^{flox/flox}$ and $Wnt2b^{\Delta/\Delta}$ mice, respectively. Genotypes were confirmed by PCR with allele-specific primers (KO1: 5'-GCC TCT CAC ACC AGC GTG TAA GAG-3', KO4: 5'-GTA ATT GAG TGG TCT CCA CC-3', Neo3': 5'-ATCAGC AGCCTC TGT TCC ACA TAC-3', PJL: 5'-TGC TAA AGC GCA TGC TCC AGA CTG-3', 2b-1: 5'-AGG AGT CTG CTC CTG ATT ACA GCC-3') to detect WT (200 bp fragment), flox (260 bp) or Δ (320 bp) allele (Fig. 1A and C).

Intercrosses of $Wnt2b^{WT/flox}$ or $Wnt2b^{WT/\Delta}$ mice produced $Wnt2b^{flox/flox}$ or $Wnt2b^{\Delta/\Delta}$ (*Wnt2b* KO) mice in the expected Mendelian ratios (Table 1). $Wnt2b^{\Delta/\Delta}$ mice appeared normal (Fig. 2A) and healthy during their lifespan (not shown). These results indicate that inactivation of *Wnt2b* did not affect body plan formation, despite *Wnt2b* being strongly and specifically expressed in the PS during gastrulation [28]. Indeed, several canonical Wnts, including *Wnt3*, *3a*, and *8a*, are co-expressed with *Wnt2b* in the primitive streak (PS) [25] and therefore could functionally compensate for the absence of *Wnt2b*. Only *Wnt3* and *Wnt3a* are required for A-P axis formation and extension [10, 15, 22, 25, 26], suggesting that they are the compensatory genes during gastrulation in $Wnt2b^{\Delta/\Delta}$ animals. Intercrosses of $Wnt2b^{\Delta/\Delta}$ mice led to viable $Wnt2b^{\Delta/\Delta}$ progeny (Table 1), indicating that $Wnt2b^{\Delta/\Delta}$ males and females are fertile and that there is no requirement for *Wnt2b* in the ovary where it is also expressed [20]. In addition, histological analyses indicated that limb, liver, kidney, eye and inner ear morphology was normal (not shown), despite strong *Wnt2b* expression in these

tissues [3, 4, 8, 11, 12, 14, 18, 28](Fig. 2B, C).

We next examined the brain since *Wnt2b* is expressed there during neural development. Brains isolated from $Wnt2b^{+/\Delta}$ and $Wnt2b^{\Delta/\Delta}$ mice at 4 months of age were fixed with 10% formalin, dehydrated by ethanol and xylene and then embedded in paraffin to make sections for histochemical staining. Five-μm-thick sections were stained by hematoxylin and eosin. Histochemical analysis indicated that there were no obvious patterning defects in the cerebral cortex or hippocampus (Fig. 3B). As in many other tissues, *Wnt2b* is coexpressed in the brain with several other canonical Wnts, particularly in the cortical hem, hippocampus and pineal gland [1, 21]. Surprisingly, we found that both the length (major axis) and width (minor axis) of the olfactory bulb (OfB) was significantly reduced (length, $p < 0.01$; width, $p < 0.05$, n = 3 in both $Wnt2b^{WT/\Delta}$and $Wnt2b^{\Delta/\Delta}$, Student's *t*-test) in the $Wnt2b^{\Delta/\Delta}$ brain (Fig. 3A-C). Small OfB's were observed in dissected, whole, adult $Wnt2b^{\Delta/\Delta}$ brains, as well as in sagittal sections. To clarify whether the small OfB phenotype in adult $Wnt2b^{\Delta/\Delta}$ mice arises during embryonic or postnatal development, we examined the size of the OfB in earlier-staged perinatal (P0, postnatal day 0) embryos. Contrary to the results observed in adult mice, significant size differences in OfBs isolated from perinatal $Wnt2b^{+/\Delta}$ and $Wnt2b^{\Delta/\Delta}$ mice were not observed ($Wnt2b^{WT/\Delta}$; n=4 and $Wnt2b^{\Delta/\Delta}$; n=7) , (Fig. 3D, E). These results suggest that the smaller OfB's observed in adult $Wnt2b^{\Delta/\Delta}$ mice arises postnatally. We can conceive of several possible explanations for the small OfB phenotype. A small OfB could arise indirectly, for example due to a craniofacial developmental defect

that physically constricts OfB development. Indeed, defects in nasal bone development have been observed in *Axin2* deficient mice in which Wnt/βcatenin signaling is perturbed [27]. Alternatively, a smaller OfB could be caused by impaired neuroblast cell migration into the OfB, or by impaired neurogenesis in the subventricular zone (SVZ). It is well known that the adult OfB is maintained through the recruitment of neuronal progenitor cells originating in the SVZ. These neuronal progenitors travel from the SVZ to the granule cell layer and glomerular layer of OfB along the rostral migratory stream (RMS) and differentiate into interneurons including granule cells or periglomerular cells [2, 24]. Wnt ligands secreted from the fimbrial neuroepithelium into the cerebrospinal fluid are thought to activate canonical Wnt signaling in the SVZ [13, 19]. In addition, it is also reported that loss of Sp8, which is a transcription factor expressing in postnatal SVZ and RMS and is a target gene of Wnt/βcatenin signaling leads the developmental defects in the generation and diversity of OfB interneurons [9] [23]. Although highly speculative, it is possible that loss of Wnt2b expression in fimbrial neuroepithelium leads to reduced Wnt2b in CSF and, consequently, reduced numbers of SVZ progenitors that populate the OfB. Clearly, additional studies are required to fully understand the underlying cause of the small OfB observed in adult $Wnt2b^{\Delta/\Delta}$ mice.

We have previously reported that the combined inactivation of *Wnt2b* and *Wnt2* results in the complete loss of lung progenitor cells, providing irrefutable evidence that the closely related *Wnt2* can compensate for the absence of *Wnt2b* [6]. The present results showing that

Wnt2b is not required at most of the sites at which it is expressed suggests that many other canonical Wnts can stand-in for *Wnt2b*, and that *Wnt2b* primarily plays a secondary, supportive role during embryogenesis.

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Figure Legends

Figure 1. Generation of *Wnt2b* conditional knockout mice. (A) Schematic of gene targeting for the *Wnt2b* locus. B, *Bam*HI; E1, *Eco*RI; K, *Kpn*I; S, *Spe*I; X, *Xba*I. (B) Southern hybridization for *Wnt2b*-targeted ES cells. Both probes for the 5' locus and 3' locus were used for screening. (C) Genotyping for WT, flox and Δ alleles of the *Wnt2b* locus by PCR. Positions of primers to distinguish WT, flox and Δ alleles of the *Wnt2b* locus are indicated in A. All WT, flox and Δ alleles were typed with single PCR reaction (3-primer mix PCR) using primers KO1, KO4 and Neo3'.

Figure 2. Normal appearance of *Wnt2b* mutant mice. (A) $Wnt2b^{WT/\Delta}$ (agouti coat color) and $Wnt2b^{\Delta/\Delta}$ (albino coat color) mice are shown. (B-C) Eye (B) and limbs (C) of $Wnt2b^{WT/WT}$, $Wnt2b^{WT/\Delta}$ and $Wnt2b^{\Delta/\Delta}$ mice are indicated. FL, forelimb; HL, hindlimb.

Figure 3. Postnatal development of the brain in *Wnt2b* mutant mice. (A) Adult brains isolated from 4 month old $Wnt2b^{WT/\Delta}$ and $Wnt2b^{\Delta/\Delta}$ mice are shown. Scale bar represents 5 mm. (B) 4 month old $Wnt2b^{flox/flox}$ and $Wnt2b^{\Delta/\Delta}$ brains were sectioned sagitally (5 μm) and stained with hematoxylin and eosin. (C) Measurements of adult OfBs (length, distance between solid white lines; widths, measured between two dashed lines in Fig. 3A) in $Wnt2b^{WT/\Delta}$ control (n=3) and $Wnt2b^{\Delta/\Delta}$ (n=3) mice are indicated. (D) Brains isolated from perinatal $Wnt2b^{WT/\Delta}$ and $Wnt2b^{\Delta/\Delta}$ mice are shown. Scale bar indicates 5 mm. (E) Measurements of OfBs isolated from perinatal $Wnt2b^{WT/\Delta}$ (n=4) and $Wnt2b^{\Delta/\Delta}$ (n=7) mice

are indicated. OfB, olfactory bulb; Hp, hippocampus; SVZ, subventricular zone.

Table 1

Intercross	No. of pups (%)			Total
	WT/WT	WT/flox	flox/flox	
Wnt2b$^{WT/flox}$				44
	13 (29.5)	19 (43.2)	12 (27.2)	
	WT/WT	WT/Δ	Δ/Δ	
Wnt2b$^{WT/\Delta}$				94
	30 (31.9)	40 (42.6)	24 (25.5)	
	WT/WT	WT/Δ	Δ/Δ	
Wnt2b$^{\Delta/\Delta}$				14
	0 (0)	0 (0)	14 (100)	

Table 1. *Wnt2b* mutant mice were intercrossed with each other, and then the genotypes of offsprings obtained from the intercrossing were determined by PCR (shown in Fig. 1A and C) at 3 weeks of age. The numbers and ratios of each genotype are indicated.

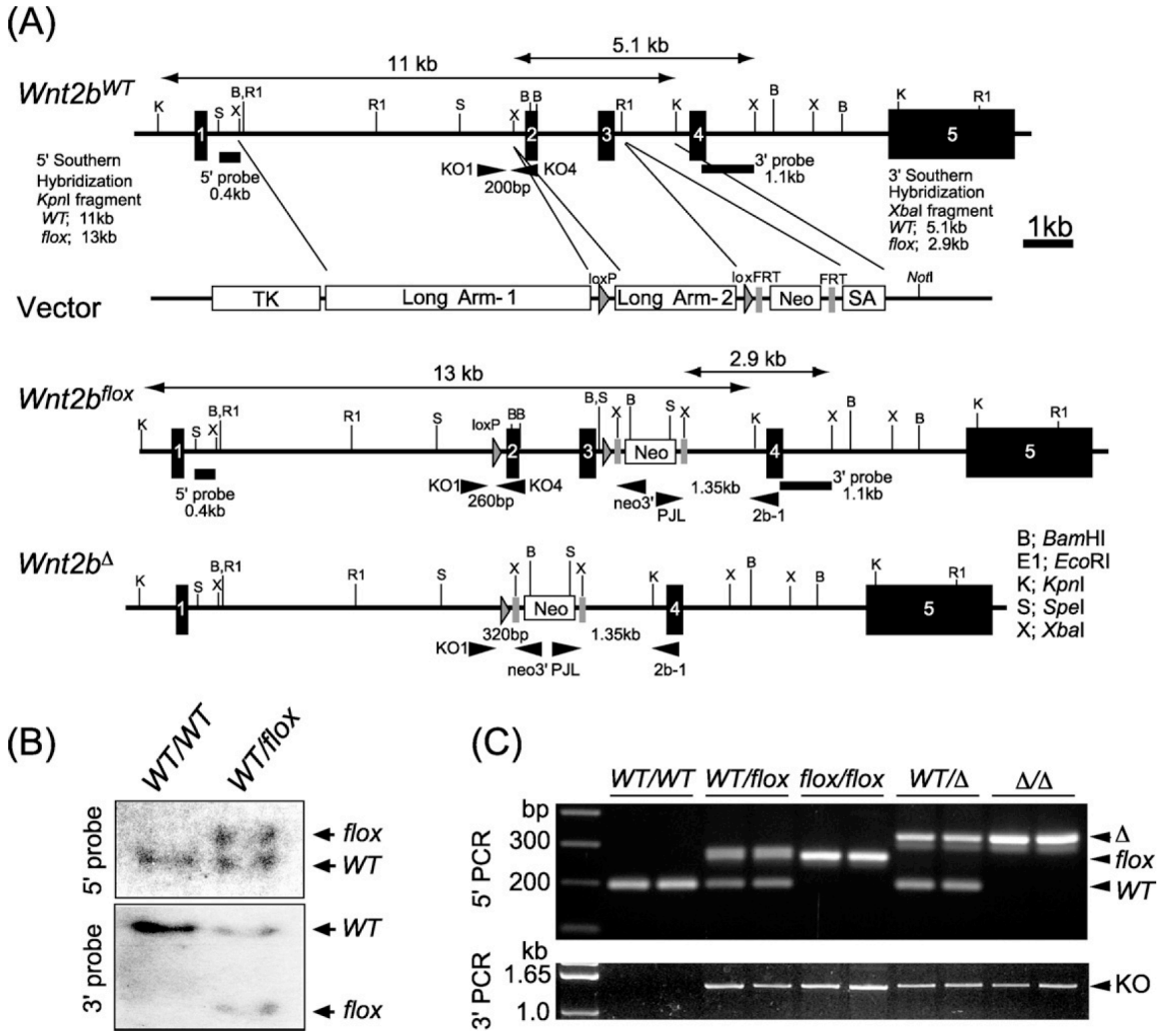
(A)
Wnt2b^WT
11 kb
5.1 kb
5' Southern Hybridization KpnI fragment WT; 11kb flox; 13kb
5' probe 0.4kb
KO1
KO4
200bp
3' probe 1.1kb
3' Southern Hybridization XbaI fragment WT; 5.1kb flox; 2.9kb
1kb
Vector
TK
Long Arm-1
loxP
Long Arm-2
loxFRT
Neo
FRT
SA
NotI
Wnt2b^flox
13 kb
2.9 kb
260bp
neo3'
PJL
1.35kb
2b-1
Wnt2b^Δ
320bp
B; BamHI
E1; EcoRI
K; KpnI
S; SpeI
X; XbaI
(B)
WT/WT
WT/flox
5' probe
3' probe
flox
WT
(C)
WT/WT
WT/flox
flox/flox
WT/Δ
Δ/Δ
bp
300
200
5' PCR
kb
1.65
1.0
3' PCR
Δ
KO

Figure 1

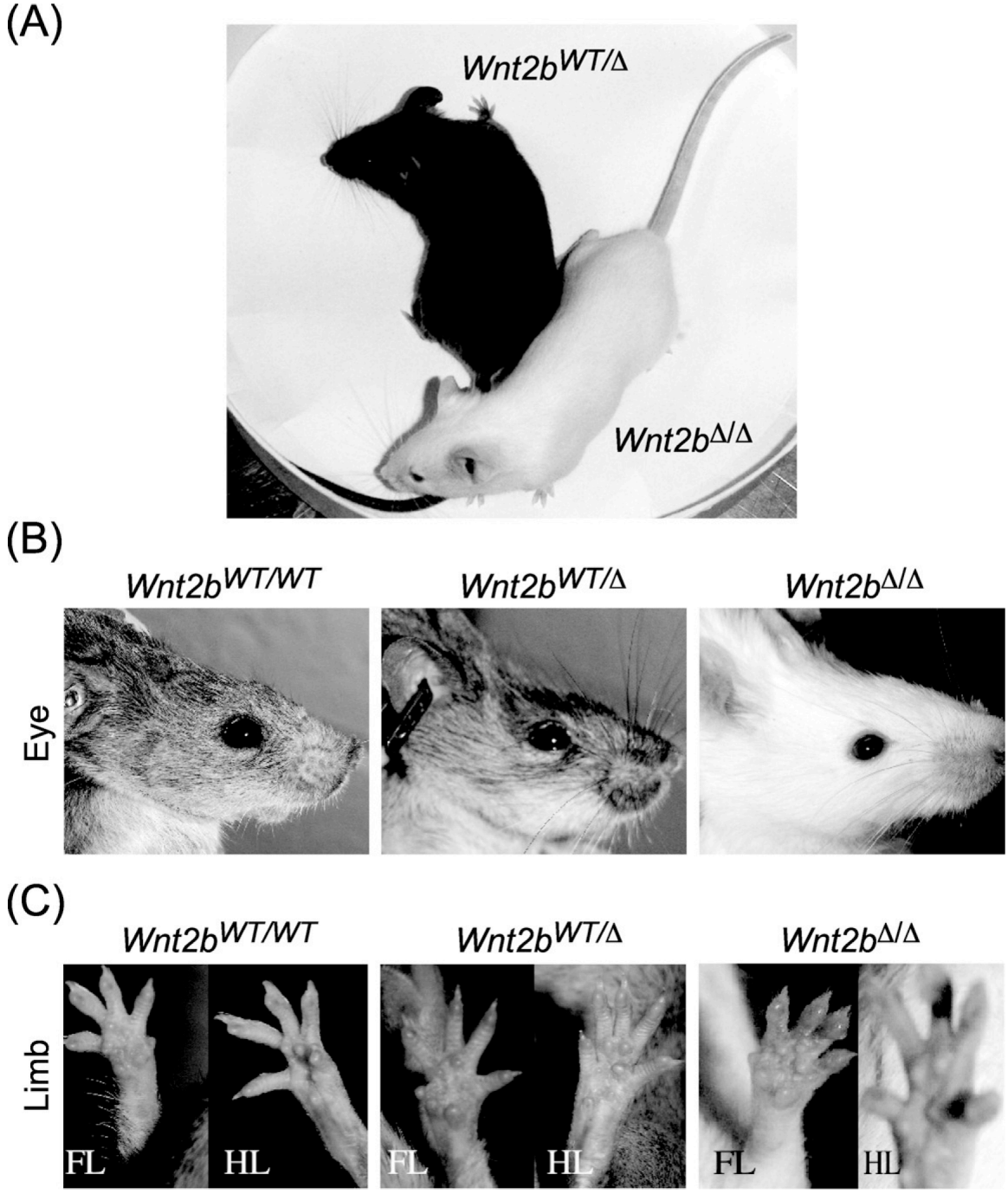
(A)
Wnt2b^WT/Δ
Wnt2b^Δ/Δ
(B)
Wnt2b^WT/WT
Wnt2b^WT/Δ
Wnt2b^Δ/Δ
Eye
(C)
Wnt2b^WT/WT
Wnt2b^WT/Δ
Wnt2b^Δ/Δ
Limb
FL
HL
FL
HL
FL
HL

Figure 2

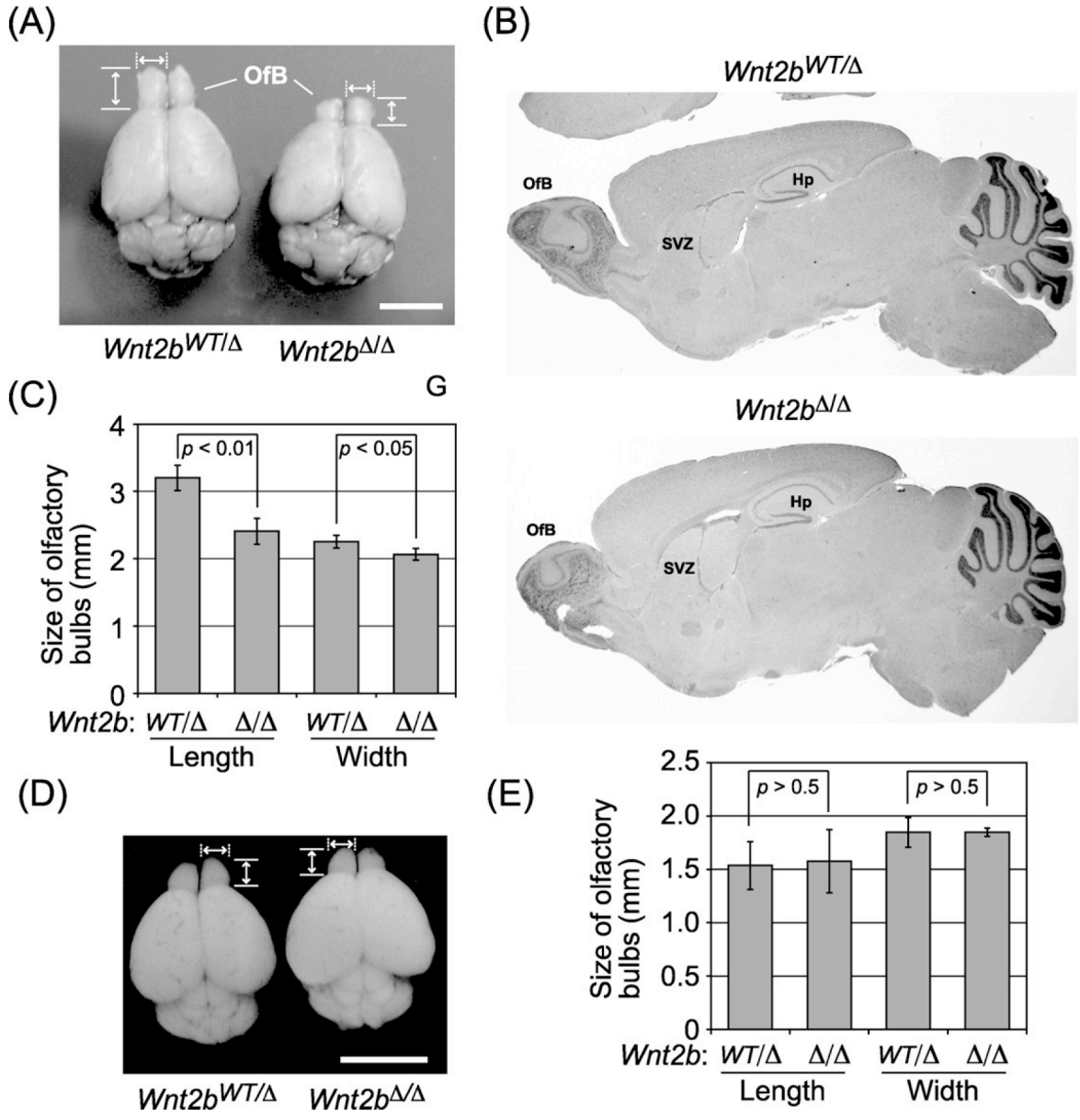
(A)
OfB
Wnt2b^WT/Δ
Wnt2b^Δ/Δ
(B)
Wnt2b^WT/Δ
OfB
Hp
SVZ
Wnt2b^Δ/Δ
OfB
Hp
SVZ
(C)
G
Size of olfactory bulbs (mm)
p < 0.01
p < 0.05
Wnt2b: WT/Δ Δ/Δ WT/Δ Δ/Δ
Length
Width
(D)
Wnt2b^WT/Δ
Wnt2b^Δ/Δ
(E)
Size of olfactory bulbs (mm)
p > 0.5
p > 0.5
Wnt2b: WT/Δ Δ/Δ WT/Δ Δ/Δ
Length
Width

Figure 3