A switch from canonical to noncanonical Wnt signaling mediates early differentiation of human neural stem cells.

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Abstract

Wnt/ β -catenin signaling is essential for neurogenesis but less is known about β -catenin-independent Wnt signals. We show here that Wnt/AP-1 signaling drives differentiation of human embryonic stem (hES) cell and induced pluripotent stem (iPS) cell-derived neural progenitor cells. Neuronal differentiation was accompanied by a reduction in β -catenin/Tcf-dependent transcription and target gene expression, increased levels and/or phosphorylation of ATF2, CREB and c-Jun and increased AP-1-dependent transcription. Inhibition of Wnt secretion using the porcupine inhibitors IWP-2 and Wnt-C59 blocked neuronal differentiation, while activation or inhibition of Wnt/ β -catenin signaling had no effect. Neuronal differentiation increased expression of several Wnt genes, including WNT3A, silencing of which reduced differentiation. Addition of recombinant Wnt-3a to cells treated with IWP-2 or Wnt-C59 increased AP-1 levels and restored neuronal differentiation. The effects of Wnt-3a could not be blocked by addition of Dkk-1 or IWR-1, suggesting the involvement of noncanonical signaling. Consistent with this, restoration of neuronal differentiation by Wnt-3a was reduced by inhibition of Jun N-terminal kinase (JNK) and by gene silencing of ATF2. Together, these observations suggest that β -catenin-independent Wnt signals promote NSC differentiation in a signaling pathway involving Wnt-3a, JNK and ATF2.

INTRODUCTION

Wnt proteins play roles in many cellular and physiological processes, regulating cell proliferation, differentiation, migration and patterning during development and tissue homeostasis in adults, primarily through their effects on stem/progenitor cells [1, 2]. In the canonical Wnt pathway, β -catenin is degraded by a protein complex containing Axin, glycogen synthase kinase-3 (GSK-3), casein kinase 1 (CK1) and adenomatous polyposis coli (APC). When a Wnt protein binds to frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP5/6) receptors, Axin and disheveled (DVL) are recruited to the membrane, leading to disruption of the degradation complex, β -catenin stabilization and entry into the nucleus and activation of T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) target genes [1]. In addition, noncanonical Wnt signals affect DVL and other intracellular components to activate the Planar Cell Polarity (PCP) and Wnt-Calcium pathways [3]. In the PCP pathway, FZD receptors activate a cascade that involves the small GTPases and c-Jun N-terminal kinase (JNK), leading to changes in the cytoskeleton and activation of Activator Protein-1 (AP-1) family transcription factors, including activating transcription factor 2 (ATF2), c-Jun and cyclic AMP response element-binding protein (CREB) [4]. Noncanonical Wnt ligands interact with numerous receptors, including receptor tyrosine kinase-like orphan receptor (ROR1/2), receptor-like tyrosine kinase (RYK) and van gogh-like (VANGL1/2) [5]. Adding to this complexity, the Wnt response can differ depending on the cellular or Wnt receptor context [6, 7], further underlining the importance of knowing the 'Wnt status' of cells at different stages of differentiation.

Many studies highlight the critical role of Wnt signaling in neural differentiation, but the details remain unclear, with reports of increased and decreased Wnt/ β -catenin signaling, as well as changes in noncanonical Wnt signaling, being required [8, 9]. The importance of

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Wnt/ β -catenin signaling may lie in its role in maintaining neural stem/progenitor cell (NSC) proliferation: inhibition of Wnt/ β -catenin signaling reduces NSC proliferation in neurospheres [10] and loss of the secreted Wnt antagonist Dickkopf-1 (Dkk-1) increases the number of hippocampal neural progenitors [11]. Constitutive activation of Wnt/ β -catenin signaling, however, disrupts NSC proliferation, migration and differentiation [12, 13]. Moreover, basal Wnt/ β -catenin signaling has been shown to repress neuronal differentiation of human NSCs [14], and its inhibition promotes neural precursor specification [15]. Thus, tight control of Wnt/ β -catenin signaling is important for proper differentiation. Comparatively little is known about noncanonical Wnt signaling in this context [16]. Wnt-5a has been shown to cooperate with Wnt-1 to regulate morphogenesis and neurogenesis during the generation of mouse midbrain dopaminergic (DA) neurons [17], and Wnt-4 and Wnt-11 play distinct roles in neuronal differentiation of NT2 cells [9]. The downstream effectors of these Wnts are not known, but candidates include ATF2 and CREB, which are implicated in neurogenesis [18, 19].

The generation of new neurons from NSCs takes place in the subventricular zone and in the subgranular zone of the hippocampus [20]. Wnt signaling promotes hippocampal neurogenesis by stimulating neuroblast proliferation and instructing neuronal fate [21] (reviewed in [16]). Since aberrant neurogenesis is believed to be involved in epilepsy, stroke and neurodegenerative disease [22], a better understanding of the roles of Wnt signaling in NSCs can lead to new treatments for these disorders. Here we have examined Wnt signaling in human embryonic stem cell-derived neural progenitor cells, which resemble primary NSCs and have been used to study human NSC differentiation [23–25], and also in human induced pluripotent stem (iPS) cell-derived neural progenitors, which have potential uses in cell transplantation therapy, modeling of human disease and drug development [26]. Our results indicate that a switch from Wnt/ β -catenin to Wnt/AP-1 signaling takes place during NSC

differentiation. We further find that inhibition of Wnt secretion blocks neuronal differentiation, and that its restoration by Wnt-3a involves activation of JNK/ATF2. Our observations are consistent with a model in which noncanonical Wnt signaling, rather than Wnt/ β -catenin signaling, promotes human NSC differentiation.

MATERIALS AND METHODS

Cell culture and drug treatments

Human neural progenitor (hNP) cells (Aruna Biomedical, Athens, GA) were grown at 37°C, 5% CO₂ in Neurobasal Medium with 1 x B27 Supplement, 1 x Penicillin/Streptomycin and 2 mM L-Glutamine (Gibco) plus 20 ng/ml basic Fibroblast Growth Factor (bFGF) (Life Technologies) and 10 ng/ml Leukemia Inhibitory Factor (ProSpec) on Matrigel-coated plates. To coat plates, Matrigel (BD) was thawed on ice and diluted in cold Neurobasal Medium 1:200 (8.95 mg/ml), applied to pre-cooled plates and left overnight at 4°C. Plates were then washed with PBS, left in a laminar flow hood to dry for 15-20 min and then stored at 4°C for up to two weeks. Before use, plates were rehydrated with PBS for 30 min at 37°C, 5% CO₂. Cells were fed every other day and the day after plating and were passaged at 1:2 or 1:3 and used for up to 20 passages. Differentiation was induced by removal of bFGF from the medium. Unless stated otherwise, cells were differentiated for 3 days. Human iPS cell-derived neural progenitor cells (EMD Millipore, Billerica, MA) were grown on Matrigel-coated plates in ENStem-A™ Neural Expansion Medium (Millipore) with Penicillin/Streptomycin and L-Glutamine (as above) and 40 ng/ml bFGF. Cells were fed every other day and the day after plating and were passaged at 1:2 or 1:3 and used for up to 5 passages. Differentiation was induced by culture in Neurobasal Medium with 1 x B27 Supplement, Penicillin/Streptomycin, 2 mM L-Glutamine and 200 μ M Ascorbic Acid (Sigma), as above. Unless stated otherwise, cells were treated every day for 5 days. IWP-2 (Calbiochem) was used at 2 μ M, diluted in

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medium and filtered before use. Equal amounts of DMSO (Sigma) were diluted in medium and filtered before use as controls. CHIR99021 (Calbiochem), iCRT14 (Tocris), IWR-1-endo (Calbiochem) and JNK Inhibitor VIII (Millipore) and corresponding volumes of DMSO as a control, were diluted in medium prior to use at 500 nM, 1 μ M, 10 μ M and 10 μ M, respectively. Wnt-C59 (Cellagen Technology) was resuspended in DMSO, diluted in medium, and used at 100 nM or as indicated in each experiment. Rho kinase inhibitor Y-27632 (Abcam) and CAMKII inhibitor myr-AIP (Millipore) were solubilized in water and used as indicated. Recombinant Wnt-7a, Wnt-5a, Wnt-3a and Dkk-1 (R&D Systems) were used at 400 ng/ml, 200 ng/ml, 100/200 ng/ml and 100 ng/ml, respectively, with corresponding dilutions of vehicle (1% CHAPS) as a control. If not stated otherwise, cells were treated every day for 3 days; for longer treatments cells were treated every other day.

RNA analysis

RNA was extracted using the PureLink RNA Micro Kit (Invitrogen), according to manufacturer's instructions. RNA purity and quantity were measured on a Nanodrop spectrophotometer and 1 μ g of total RNA was transcribed with M-MLV-RT (Invitrogen) in the presence of RNaseOUT (Invitrogen), according to manufacturer's instructions. Real-Time PCR was performed in a ViiA7 System (Applied Biosystems) using Perfecta SYBR Green SuperMix Low Rox (Quanta). To determine the mRNA fold change of the different genes, the $\Delta\Delta$ Ct quantitation method was used, with *36B4* as the housekeeping gene. The primers used are listed in Supplemental Table 1. The TaqMan® Array, Human WNT Pathway, was used as instructed by the manufacturer (Applied Biosystems).

Immunofluorescence

Coverslips were rinsed with PBS and fixed in 4% paraformaldehyde (Santa Cruz) for 20 min, washed twice with PBS, permeabilized using 0.1% Triton in PBS for 15 min and washed 3 times with PBS to remove detergent. Blocking was performed using 2% BSA, 50 mM

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glycine, 0.01% NaN₃ in PBS for 1 h. All primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Cells were then washed gently 4 times with PBS, incubated with secondary antibody in the dark for 1 h (AlexaFluor488, AlexaFluor594 or AlexaFluor647, 1:500), washed 4 times, mounted using Vectashield (Vector Labs) and stored in the dark at 4°C. Stained cells were visualized using a Leica Epifluorescence microscope and a Leica Confocal microscope. The primary antibodies used are listed in Supplemental Table 2. In order to quantify immunofluorescence, images were taken with the same exposure time consecutively without interruption using a Leica Epifluorescence microscope, and always handled as separate-channel images with ImageJ. A minimum and maximum intensity threshold was set to representatively measure the fluorescence for the channel of interest. These values were fixed for all images from the same experiment (4 random images per treatment), and the area covered by these intensities was measured and taken as the relative area of interest for each channel and image. The same was done for the DAPI channel for each image in order to obtain a ratio of the antibody and DAPI signals, which provides a measure of the stained area for that particular target protein. The 4 values per treatment were averaged and then the averages of three independent experiments were statistically processed.

Gene reporter assays and gene silencing

Cells plated in 12-well plates at 50% confluence were washed twice with OptiMEM (Gibco) to remove antibiotics and transfected with 450 ng luciferase gene reporters (Super8xTOPFlash and Super8xFOPFlash [27], AP-1-luciferase [28] and CRE-luciferase [29]), together with a constitutive Renilla luciferase reporter (pRL-tk) using Lipofectamine LTX and Plus Reagent (Invitrogen), as instructed by the supplier. The transfection mix was removed 4 hours later and fresh medium (with or without treatment) added. After 24-48 h, the cells were washed twice with PBS and lysed in 125 ul Passive Lysis Buffer (Promega).

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Luminescence was determined using the Dual-Glo Luciferase Assay System (Promega).

Signaling activities were calculated as luciferase/Renilla and TOP/FOP ratios.

Trisilencer-27 siRNA Knockdown Duplexes were obtained from OriGene (siCTRL, siATF2, siCREB, siJUN, siW3A); three siRNAs were tested for each gene (sequences are available on request). For *FZD7* and *VANGL2* ON-TARGETplus siRNA smartpools containing 4 different sequences were acquired from Dharmacon. Cells at 30-50% confluence were washed twice in OptiMEM to remove antibiotics and transfected with siRNAs (25 nM for Trisilencer siRNAs and 100 nM for smartpools) dissolved in OptiMEM using Lipofectamine RNAiMAX (Invitrogen), as described by the manufacturer. 24h after the addition of the transfection mix, the liposomes were removed and replaced with fresh medium.

Western Blotting (WB)

Total cell extracts were obtained by lysing the cells in radio-immunoprecipitation assay (RIPA) lysis buffer (Millipore) with 1 mM EDTA (Sigma), Complete Mini EDTA-free Protease Inhibitors (Roche), PhosphoStop (Roche) and 0.1% SDS (Gibco) and centrifuged for 12 min at 15,000 g. The supernatants were combined with 2 x Laemmli Buffer (Sigma) and heated at 95°C or stored at -80°C. Nuclear and non-nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) with Complete Mini EDTA-free Protease Inhibitors (Roche). Proteins were separated on SDS polyacrylamide gels and transferred to PVDF membranes (Millipore). Membranes were washed in TBST (TBS 0.05% Tween (Sigma)) and incubated in blocking buffer (3% BSA (Sigma) or 5% milk in TBST) for 1 h. Primary antibodies were incubated as described in Supplemental Table 3, washed 4 times in TBST, incubated for 1 h in blocking buffer with HRP-conjugated secondary antibodies diluted at 1:10,000, washed again and developed using chemiluminescence (Clarity Western ECL Substrate, BioRad).

Statistics

Three independent experiments were performed for each panel, unless otherwise stated. Students *t* test or ANOVA was applied to determine significance, as required. Error bars show SD or SEM.

RESULTS

Neuronal differentiation of human neural progenitor cells correlates with a switch from Wnt/ β -catenin to noncanonical Wnt signaling.

Characterization of hES-derived neural progenitor cells indicated that undifferentiated cells express the neural stem cell markers SOX2 and Nestin and do not express the pluripotency marker Nanog (Fig. S1A-C), confirming their commitment to the neuroectodermal lineage. Neuronal differentiation was monitored by induction of the early neuronal marker PAX6 at 1 day (Fig. S1F) and increasing expression of NEUROD1 (ND1, Fig. S1D) and β -III tubulin (Fig. S1C). In addition, we noted an increase in the intensity of β -catenin staining at the plasma membrane in differentiating cells (Fig. S1E). Wnt signaling activity in undifferentiated and differentiating hNP cells was then measured using gene reporter assays (Fig. 1). Wnt/ β -catenin signaling was significantly lower in differentiating cells, as indicated by a reduction in Super8xTOPFlash reporter activity; the control Super8xFOPFlash reporter with mutations in the Tcf/LEF binding sites was not affected (Fig. 1A). Noncanonical Wnt signals activate AP-1 transcription factors, particularly ATF2 [30, 31]. ATF2-luciferase provides a readout for noncanonical Wnt signaling in amphibians but not in mammalian cells [32]. However, AP-1 family transcription factor activity can be measured using AP-1- and CRE-luciferase reporters. Both AP-1- and CRE-luciferase activities increased upon neuronal differentiation (Fig. 1B, C). *In addition, the phosphorylation and nuclear localization of ATF2 and CREB, which are induced by noncanonical Wnt signals in other cell types [31, 33, 34],*

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increased during differentiation (Fig. 1D-G), whereas the level of nuclear β -catenin did not change (Fig. 1G). In summary, neural differentiation is accompanied by a reduction in Wnt/ β -catenin signaling and increases in AP-1- and CRE-luciferase activities and ATF2/c-Jun/CREB protein levels, suggestive of an increase in noncanonical Wnt signaling.

In order to determine if this apparent switch in Wnt signaling could be observed at the mRNA level, we examined expression of Wnt pathway components using a real-time PCR TaqMan array. Neuronal differentiation was verified by real-time PCR analysis of the expression of the neuronal genes *NEUROD1* (*NDI*) and *ASCL1* (Fig. S2A). The top twelve up- and down-regulated Wnt pathway genes included several noncanonical Wnts, whose expression increased during differentiation (Fig. S2B). There were fewer changes among the Wnt receptors, but *FZD7* expression increased and *FZD6* expression was reduced during differentiation. In addition, there was a reduction in the expression of the Wnt target genes *NKDI*, *DKK1* and *MYC*, but not of *AXIN2*. Three independent time-course experiments using conventional real-time PCR were used to confirm these results (Fig. 2). There was a correlation between increased expression of the early neural genes *ASCL1*, *NDI* and *NGN2*, increased expression of *WNT3A*, *WNT4*, *WNT5A*, *WNT5B*, *WNT7A*, *WNT7B* and *FZD7* and reduced expression of *FZD6*, *DKK1*, *MYC* and *NKDI* during differentiation (Fig. 2A-D). Since the upregulated Wnt genes predominantly activate the noncanonical pathway in neural cells [9, 35, 36], these results are consistent with the gene reporter assays showing a reduction in Wnt/ β -catenin signaling and an increase in AP-1 signaling during differentiation.

An endogenous noncanonical Wnt signal is required for neuronal differentiation.

In order to determine the importance of endogenous Wnts for neuronal differentiation, we tested the impact of blocking Wnt secretion using IWP-2, which inhibits porcupine, the enzyme that palmitoylates Wnt proteins [37]. Treatment of cells with IWP-2 reduced

expression of neuronal differentiation markers both under basal conditions and during differentiation (Fig. 3A). The same results were obtained using a second porcupine inhibitor, Wnt-C59 [38] (Fig. 3C and D). IWP-2 reduced expression of the Wnt target genes *AXIN2* and *NKDI* in undifferentiated cells (Fig. 3B), indicating that they secrete and respond to canonical Wnts. In contrast, IWP-2 did not affect expression of *AXIN2* or *NKDI* in differentiating cells, consistent with their reduced canonical Wnt signaling activity. Importantly, IWP-2 reduced neuronal gene expression both in undifferentiated and differentiating cells, indicating that Wnt secretion is required for neuronal gene expression, irrespective of Wnt/ β -catenin signaling activity.

Since neuronal differentiation is often accompanied by glial differentiation, we also examined the expression of markers for astrocytic (*CD44* and *S100A* [39]) and oligodendrocytic (*OLIG1* and *PDGFRA* [40]) differentiation. Wnt-C59 treatment of undifferentiated cells reduced expression of the astrocytic markers and increased expression of the oligodendrocytic markers (Fig. S2D), suggesting that endogenous Wnts affect basal expression of glial markers in undifferentiated cells. However, induction of hNP differentiation was accompanied by a strong reduction in the expression of these markers, indicating that these culture conditions promote neuronal rather than glial differentiation (Fig. S2C). Moreover, treatment of cells with Wnt-C59 did not affect glial marker expression in differentiating cells, as expected given the already reduced levels of these genes (Fig. S2D).

To determine if changes in Wnt/ β -catenin signaling directly affect neuronal differentiation, we used iCRT-14, which inhibits Wnt/ β -catenin signaling by blocking the interaction between β -catenin and TCF4 [41], and the GSK-3 inhibitor CHIR99021, which activates Wnt/ β -catenin signaling by preventing β -catenin degradation [42]. Treatment of cells with iCRT-14 and CHIR99021 induced a 2-fold reduction (Fig. 3E) and a 3-fold increase (Fig. 3F) in

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Wnt/ β -catenin activity, respectively. However, neither compound affected neuronal gene expression (Fig. 3G). Together, these results strengthen the case for noncanonical rather than Wnt/ β -catenin signaling playing a role in neuronal differentiation.

Noncanonical Wnt-3a signaling restores neuronal differentiation of hNP cells.

To determine if exogenous Wnt proteins could rescue the effects of IWP-2, recombinant Wnts were added to IWP-2-treated hNP cells during differentiation. Wnt-3a, Wnt-7a and Wnt-5a (Fig. 4A-B and Fig. S2E) rescued neuronal gene expression in IWP-2-treated cells. Wnt-7a and Wnt-5a were much less effective than Wnt-3a, which completely restored neuronal gene expression to levels in differentiating control cells. This result was unexpected, since Wnt-3a normally strongly activates β -catenin, but is consistent with the increase in endogenous *WNT3A* mRNA during differentiation (Fig. 2B). We therefore explored the function of Wnt-3a in more detail, hypothesizing that it rescues neuronal gene expression by activating a noncanonical signal. To test this possibility, we used Dkk-1, a secreted Wnt antagonist that binds to LRP5/6 Wnt co-receptors, thereby inhibiting canonical but not noncanonical Wnt signaling [1]. We surmised that if Wnt-3a rescues the effect of IWP-2 by activating a canonical Wnt signal, this would be prevented by Dkk-1 (Fig. S3A). As expected, Dkk-1 blocked activation of canonical signaling by Wnt-3a, as measured by inhibition of β -catenin/Tcf target gene expression (Fig. 4C). The results of gene reporter assays further demonstrated efficient inhibition of canonical Wnt signaling by Dkk-1 (Fig. S3B). However, Dkk-1 did not block the effects of Wnt-3a on neuronal gene expression (Fig. 4D), indicating that Wnt-3a promotes neuronal differentiation by activating a noncanonical signal. We also noted that differentiating cells formed islands of cells that express high levels of ND1 and

doublecortin (DCX). Inhibition of Wnt secretion prevented formation of these islands, and this effect was rescued by Wnt-3a and by Wnt-3a and Dkk-1 together (Fig. 4E).

To extend these findings, we inhibited Wnt/ β -catenin signaling using IWR-1, which promotes β -catenin degradation by stabilizing Axin. As expected, IWR-1 inhibited Wnt-3a activation of β -catenin/Tcf transcriptional activity in Wnt-C59-treated cells (Fig. 5A) and Wnt/ β -catenin target gene expression (Fig. 5B). Consistent with the experiments using Dkk-1, IWR-1 did not affect neuronal gene expression (Fig. 5C). To determine if noncanonical Wnt-3a signaling is sufficient for neuronal differentiation beyond the first 3 days, cells were treated for 7 days with Wnt-3a and the same inhibitors. We then analyzed neuronal differentiation by q-PCR (Fig. 5D) and by immunofluorescence for DCX and β -III tubulin (Fig. 5E-G). Wnt-C59 inhibition of Wnt secretion completely blocked neuronal differentiation and this was rescued by recombinant Wnt-3a in the presence of IWR-1, supporting a role for noncanonical Wnt-3a signaling at later stages of differentiation.

Wnt-3a/AP-1 signaling is required for neuronal differentiation.

To determine if noncanonical Wnt-3a signals increase neuronal differentiation via AP-1 signaling, we examined the effects of Wnt-C59 on AP-1 family proteins in differentiating cells. hNP cells cultured in the presence of Wnt-C59 and treated with Wnt-3a with or without Dkk-1 were analyzed for AP-1 family proteins using immunofluorescence. Wnt-3a increased the levels of phospho-CREB, phospho-ATF2, ATF2 and c-Jun in differentiating islands of cells, and these changes were not affected by Dkk-1 (Fig. S4A-D). This further supports a model in which Wnt-3a/AP-1 rather than Wnt-3a/ β -catenin signaling promotes neuronal differentiation.

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JNK is a kinase implicated in noncanonical Wnt signaling that can phosphorylate and stabilize ATF2 and c-Jun. To determine if JNK signaling is required for neuronal differentiation, we tested the effects of the JNK-specific inhibitor JI8 [43]. JI8 reduced the levels of c-Jun, phospho-CREB, phospho-ATF2 and ATF2 (Fig. 6A), AP-1 gene reporter activity (Fig. S5A) and expression of neuronal genes (Fig. 6B). To determine if JNK activity is required for Wnt-3a induction of neuronal differentiation independently of Wnt-3a/ β -catenin signaling, we tested the effects of JI8 using cells cultured in Wnt-C59 and treated with Wnt-3a and IWR-1. JI8 was found to inhibit neuronal gene expression (Fig. 6C) without affecting Wnt/ β -catenin target gene expression (Fig. 6D), consistent with a model in which Wnt-3a/JNK signaling promotes neuronal differentiation independently of Wnt/ β -catenin.

We next used gene silencing to determine if any of the AP-1 family proteins are specifically required for neuronal differentiation. In addition, since *WNT3A* mRNA expression was observed to increase early during differentiation and from a lower baseline than other Wnt family members, we also silenced Wnt-3a. Optimal siRNAs for silencing ATF2, CREB, JUN and WNT3A were selected (Fig. 6G). Silencing Wnt-3a and ATF2, but not CREB or JUN, reduced neuronal gene and protein expression in differentiating cultures (Fig. 6E and G and S6B-C), highlighting the importance of endogenous Wnt-3a and ATF2 in neuronal differentiation of hNP cells. In order to determine if ATF2 is required for Wnt-3a restoration of neuronal differentiation, cells were transfected with siRNAs, treated with Wnt-C59 to block endogenous Wnt secretion and treated with Wnt-3a in the presence of IWR-1 to selectively activate noncanonical Wnt signaling. Silencing of ATF2 reduced expression of three of the four neuronal genes tested (Fig. 6F), indicating that ATF2 is an important downstream effector of Wnt-3a during neuronal differentiation.

Noncanonical Wnt-3a and ATF2 promote neuronal differentiation in iPS-derived neural progenitor cells.

In order to determine if Wnt-3a/AP-1 signaling is of general importance for neuronal differentiation of human stem cells, we carried out studies using human iPS cell-derived neural progenitor cells. The cells were first evaluated by immunocytochemistry and western blotting, which confirmed that they express the neural stem cell markers SOX2 and Nestin and can be induced to undergo neuronal differentiation, as indicated by expression of DCX and PAX6, which was observed at 5 days (Fig. S6A-D). We also observed increased levels of phospho-ATF2 in differentiating cells (Fig. S6C). Neuronal gene expression was measured in a 7-day time course to provide an indication of the rate of neuronal differentiation (Fig. S7A). Consistent with what was seen in hNP cells, neuronal differentiation was accompanied by increased expression of *WNT3A*, *WNT7A* and *WNT7B* (Fig. S7B), reduced expression of *DKK1* and *NKDI*, and a small increase in *AXIN2* expression (Fig. S7C). [As we previously observed in hNP cells, glial markers were also reduced upon differentiation \(Fig. S7D\).](#)

Given the similarities with hNP cells, we examined the responses of iPS cell-derived neural progenitor cells to Wnt-C59 and subsequent treatment with Wnt-3a in the presence or absence of IWR-1 to block Wnt/ β -catenin signaling. Wnt-C59 inhibited neuronal differentiation of iPS cell-derived neural progenitors, as measured by expression of DCX in cell cultures (Fig. 7A, B) and by expression of neuronal genes. Addition of recombinant Wnt-3a rescued neuronal gene expression and IWR-1 was unable to block this effect of Wnt-3a (Fig. 7C), although it did inhibit Wnt-3a-induced expression of Wnt/ β -catenin target genes (Fig. S7E). The levels of phospho-ATF2 responded similarly to these treatments; being reduced by Wnt-C59 and stimulated by Wnt-3a in the presence or absence of IWR-1 (Fig. 7F). Finally, ATF2 gene silencing reduced the number of DCX-positive cells in differentiating cultures (Fig. 7D and E), indicating that ATF2 is also important for differentiation of iPS-derived neural

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progenitors. These results are similar to those obtained using hNP cells, suggesting Wnt-3a/ATF2 signaling plays a general role in neural stem cell differentiation.

DISCUSSION

The demonstration that inhibition of Wnt secretion using the porcupine inhibitors IWP-2 and Wnt-C59 blocks neural stem cell differentiation is consistent with studies highlighting the importance of Wnt signaling in neural development [44]. However, our observations that β -catenin/Tcf activity and target gene expression fall during differentiation, and that activation and inhibition of Wnt/ β -catenin signaling do not affect neuronal differentiation, are not in keeping with a role for Wnt/ β -catenin signaling in differentiation *per se*. Inhibition of Wnt secretion had different effects on β -catenin/Tcf target gene expression in undifferentiated and differentiating cells, consistent with Wnt/ β -catenin signaling playing a role in self-renewal and/or survival of neural progenitors, rather than differentiation [10, 11]. However, we cannot rule out that Wnt/ β -catenin signaling during the self-renewal phase also primes the cells to differentiate appropriately. We also tried to determine whether Wnt signaling was implicated in the differentiation of glial cells. Inhibition of Wnt secretion in undifferentiated cells did alter basal expression of glial genes, favoring expression of oligodendrocytic over astrocytic markers. However, culture in neuronal differentiation medium reduced basal expression of glial markers both in hNP and iPS-derived NSCs, preventing further analysis of the effects of noncanonical Wnt signaling on glial differentiation

Several Wnt genes were upregulated during neuronal differentiation. Among these, *WNT3A*, *WNT7A* and *WNT7B* were strongly induced in both hNP and iPS-derived neural progenitor cells. Wnt-3a, in particular, appears to play a key role, since gene silencing of Wnt-3a alone inhibited neuronal gene expression and recombinant Wnt-3a was sufficient to restore neuronal differentiation in cells treated with porcupine inhibitors. The patterns of expression of these

Wnts suggest they play a role in mouse neural progenitors, with *WNT3A* being found in the ventricular zone of the dorsal spinal cord and the hindbrain [45] and *WNT7A* and *WNT7B* present in the ventricular zone in the ventral-lateral spinal cord and cortical forebrain [45], and *WNT7B* also expressed in the adult dentate gyrus (Fig. S8A, Supplemental online video 1) (<http://mouse.brain-map.org> [46]). Wnt-7a and Wnt-5a were less efficient than Wnt-3a at restoring neuronal differentiation in hNP cells. Nevertheless, both Wnt-7a and Wnt-5a have been demonstrated to activate noncanonical signals in neurons: Wnt-7a, for example, promotes dendritic spine growth and synaptic strength in hippocampal neurons [35], while Wnt-5a is implicated in the morphogenesis and neurogenesis of dopaminergic neurons [17]. Whether Wnt-5a and Wnt-7a/b play distinct roles in NSC differentiation, compared to Wnt-3a, remains to be determined.

Our results indicate that the noncanonical Wnt signal that drives neuronal differentiation involves JNK and AP-1 family members. As noted in the Introduction, there are several different noncanonical Wnt signaling pathways. In order to begin to address which of them might be involved in the response to Wnt-3a, we used a number of other small molecule inhibitors, but the results were inconclusive: inhibition of Rho kinase using Y-27632 did not affect expression of *NGN2* or *DCX* and increased *NDI* expression, while inhibition of CaMKII using Myr-AIP slightly reduced *NGN2* and *DCX* expression and did not affect *NDI* (Fig. S9C-D). In addition, silencing of the Wnt/PCP pathway component *VANGL2*, which was upregulated during differentiation, did not significantly affect neuronal gene expression (Fig. S9A-B). While these results do not exclude the involvement of Wnt/Calcium and Wnt/PCP signaling, the lack of consistent effects prompted us to focus on JNK/AP-1. Wnt-3a, Wnt-7a and Wnt-7b have all been shown to activate JNK [36, 47–49], which is a major AP-1 kinase [50]. Moreover, inhibition of JNK blocked the ability of Wnt-3a to restore neuronal differentiation. Neuronal differentiation of hNP cells was paralleled by increases in AP-1- and

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CRE-dependent gene reporter activities and expression and/or phosphorylation of the ATF2, c-Jun and CREB. Both ATF2 and CREB have been linked to neurogenesis in the adult mouse hippocampus [18, 51], and, according to data from the Allen Brain Atlas, both are highly expressed in the dentate gyrus (Fig. S8B, C and supplemental online video 2 (*Atf2*)), supporting a role for these proteins in differentiation of adult NSCs *in vivo*. Our gene silencing experiments indicate that ATF2 is required for neuronal gene expression in both hNP and iPS-derived neural progenitor cells. However, there is considerable crosstalk among AP-1 family members, so roles for c-Jun and CREB should not be ruled out.

Since Wnt-3a signals can both maintain mouse neural stem/progenitor cells [52] and promote neural differentiation [53], the response to Wnt-3a is likely to depend on the Wnt receptors expressed and/or the presence of other factors that affect Wnt signaling, such as secreted Wnt antagonists. Dkk-1, for example, has been reported to suppress neural progenitor proliferation [11] and sFRP3 inhibits quiescent NSC activation and neural progenitor maturation [54]. However, abrogation of sFRP3, but not Dkk-1 expression, increases the number of mature neurons. This supports a model in which noncanonical Wnt signals play a role in neuronal differentiation, since only sFRP3 inhibits both canonical and noncanonical signaling. We have used Dkk-1 as a tool to block canonical Wnt signaling and thereby reveal the noncanonical signaling function of Wnt-3a. We obtained similar results using IWR-1 to inhibit canonical signaling both in hNP and iPS-derived neural progenitor cells, further supporting the view that β -catenin signaling activity is not required for the effect of Wnt-3a. Noncanonical Wnt-3a signaling has been observed in mesenchymal stem cells, where it inhibits chondrogenesis via activation of calcium/calmodulin-dependent kinase II (CaMKII) [55], and in Ewing tumor cells, where Wnt-3a and Dkk-1 induce neurite outgrowth [47]. However, to our knowledge, this is the first report describing a noncanonical role for Wnt-3a in hES and iPS-derived neural progenitor cells.

The mechanism responsible for the switch in the response to Wnt-3a remains unclear. Differentiation is initiated by the removal of FGF2. FGF2 has been reported to increase canonical Wnt signaling via inhibition of GSK-3 [56], so its removal may be responsible for the observed reduction in Wnt/ β -catenin signaling. The switch to noncanonical signaling could result from changes in expression of inhibitors or effectors of Wnt canonical and noncanonical signaling. Neuronal differentiation of hNP cells is accompanied by a fall in expression of both *DKK1* and *DKK2*, so Dkk proteins are unlikely to be involved. One possibility is that it is mediated by changes in Wnt receptor expression. Our analysis of Wnt receptor mRNA expression levels did not reveal many changes apart from increased expression of *FZD7* (Fig. 2C and S2B). However, when we silenced *FZD7* there was no effect on neuronal gene expression (Fig. S9A-B). A complete answer will require silencing combinations of candidate Wnt receptors. In addition, differentiation may be accompanied by posttranscriptional changes in the Wnt receptor repertoire, a prospect that deserves further investigation.

Another possibility is that the switch involves changes in the relative expression levels of Tcf/LEF and AP-1 family transcription factors. A recent report showed that RA-induced differentiation of mES cells is accompanied by a reduction in Tcf-1 and an increase in Tcf-3 expression, with the latter repressing canonical Wnt signaling and promoting noncanonical signaling and differentiation [57]. We did not observe changes in the expression of TCF (Tcf-1) in the Wnt array analysis, but there was a 1.7-fold increase in expression of TCF7L1 (Tcf-3) upon differentiation (unpublished observations), so it is possible that Tcf-3 plays a role in the switch. Alternatively, the switch could result from increased expression of AP-1 family members, particularly ATF2. ATF2 has been reported to bind to Tcf family proteins in hematopoietic tumor cells, leading to activation of a Tcf reporter [58]. While this may be inconsistent with the reduction in Tcf reporter activity we observed in differentiating neural

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stem cells, the transcriptional activity of ATF2/Tcf complexes may be context-dependent.

Given the therapeutic potential of human neural stem cells, future studies directed at

clarifying the mechanism responsible for the switch may prove fruitful.

Conclusion

In summary, our results indicate that neuronal differentiation is accompanied by a reduction in canonical Wnt signaling, and, in addition, highlight the importance of noncanonical Wnt signaling in this process in two independent stem cell models. Manipulation of Wnt signaling with purified Wnt proteins is used for generating neurons [17] and other cell types [59] from stem cells. A better understanding of how the cellular response to Wnt proteins switches from canonical to noncanonical during differentiation may therefore be important for advancing stem cell-based cell replacement therapies.

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Disclosure of potential conflicts of interest

The authors declare they have no competing interests.

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Figure legends

Fig. 1. A switch from canonical to noncanonical Wnt signaling in hNP cells. (A-C) Gene reporter assays measuring β -catenin/Tcf- (A), AP-1- (B) and CRE- (C) dependent transcriptional activities in undifferentiated and differentiating cells at 48 h; * $p < 0.05$ by t test (error bars represent SD). (D-F) Immunofluorescence staining for phospho-ATF2 (D), ATF2 (E) and c-Jun (F) in undifferentiated and differentiating hNP cells; DAPI staining of nuclei is in blue. Scale bars 100 μ m. (G) Western blots for the indicated antigens in nuclear (NUC) and cytoplasmic (CYT) extracts from hNP cells at 1, 2 and 3 days of differentiation; β -cat top short exposure, bottom long exposure; lamin A (LAM A) and HSP60 were used as loading controls.

Fig. 2. Neuronal and Wnt pathway gene expression during neuronal differentiation of hNP cells. Graphs show average mRNA expression determined by q-PCR of the genes indicated, relative to *36B4*, from 3 independent 5 day time-course experiments (error bars represent SEM). *WNT3A* mRNA could not be detected at day 0, therefore graph B is normalized to day 1 whereas A, C and D are normalized to day 0.

Fig. 3. Inhibition of Wnt secretion blocks neuronal differentiation while inhibition of Wnt/ β -catenin signaling does not. (A, B) mRNA expression determined by q-PCR of the genes indicated, using undifferentiated hNP cells (UNDIF) or cells differentiated for 3 d (DIF) in the presence of vehicle (DMSO) or IWP-2 (2 μ M). (C, D) mRNA expression determined as in (A) in undifferentiated (UNDIF) or differentiating (DIF) cells treated with vehicle or the indicated concentrations of Wnt-C59. (E, F) Gene reporter assays measuring β -catenin/Tcf-dependent transcriptional activity (TOPFlash/FOPFlash ratio) in differentiating hNP cells treated with CHIR (E) or iCRT14 (F) for 24 h. (G) mRNA expression determined by q-PCR of the genes indicated, using differentiating cells treated with vehicle (DMSO),

CHIR99021 (CHIR, 0.5 μ M) and iCRT14 (1 μ M). (A, B, E and F) * $p < 0.05$ by t test, (C, D and G) * $p < 0.05$ by ANOVA (error bars represent SD).

Fig. 4. Noncanonical Wnt-3a promotes early neuronal differentiation. (A, B) mRNA expression determined by q-PCR using differentiating cells treated with vehicle (DMSO and CHAPS), IWP2 and Wnt-7a (400 ng/ml) (A) or Wnt-3a (200 ng/ml) (B). (C, D) mRNA expression determined by q-PCR of the genes indicated, using differentiating hNP cells treated as indicated with vehicle (DMSO and CHAPS), IWP2 (2 μ M), Wnt-3a (100 ng/ml) and/or Dkk-1 (100 ng/ml). (E) Immunofluorescence staining for DCX and ND1 in differentiating cells treated Wnt-C59 (100 nM) and vehicle (CHAPS), recombinant Wnt-3a (100 ng/ml), recombinant Dkk-1 (100 ng/ml) and vehicle (CHAPS) or both together; DAPI staining of nuclei is in blue, scale bars 200 μ m. * $p < 0.05$ by ANOVA (error bars represent SD).

Fig. 5. Noncanonical Wnt-3a promotes neuronal differentiation at 7 days of differentiation. (A) β -catenin/Tcf activity in cells treated for 24 h with Wnt-C59 and vehicle, IWR-1 and/or Wnt-3a. (B, C) mRNA expression in differentiating cells cultured in Wnt-C59 and treated with vehicle, IWR-1 (10 μ M) and/or Wnt-3a (100 ng/ml). (D) mRNA expression in differentiating cells cultured in vehicle, Wnt-C59 (100 nM) and vehicle, Wnt-C59 (100 nM), Wnt-3a (100 ng/ml) and vehicle, and Wnt-C59 (100 nM), Wnt-3a (100 ng/ml) and IWR-1 (10 μ M) for 7 days. (E) Immunofluorescence staining for DCX and β -III tubulin in differentiating cells treated for 7 days with vehicle (DMSO and CHAPS), Wnt-C59 (100 nM) and vehicle (CHAPS), Wnt-C59 (100 nM) and recombinant Wnt-3a (100 ng/ml), and Wnt-C59 (100 nM), recombinant Wnt-3a (100 ng/ml) and IWR (10 μ M); DAPI staining of nuclei is in blue; scale bars 200 μ m. (F, G) Relative quantification of DCX and β -III tubulin; * $p < 0.05$ by ANOVA (error bars represent SD).

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Fig. 6. Wnt-3a restoration of neuronal differentiation is blocked by inhibition of JNK and by silencing of ATF2. (A) Western blots detecting the indicated antigens in total extracts of hNP cells cultured with vehicle (DMSO) or JNK inhibitor JI8 (10 μ M); GAPDH and HSP60 were used as loading controls. (B) mRNA expression determined by q-PCR of the genes indicated, using differentiating cells treated with vehicle or JI8. (C, D) mRNA expression determined as in (B) using differentiating cells treated with Wnt-C59 (100 nM), IWR-1 (10 μ M), Wnt-3a (100 ng/ml) and vehicle or JI8; * $p < 0.05$ by t test (error bars represent SD). (E, G) mRNA expression determined as in (B) and western blots using differentiating cells silenced for 72 h and compared to their respective controls depicted as a dashed line set at 1; * $p < 0.05$ by t test (error bars represent SD). (F) mRNA expression using differentiating cells transfected with control (siC) or ATF2 siRNAs for 48h and treated with Wnt-C59 (100 nM), IWR-1 (10 μ M) and Wnt-3a (100 ng/ml) for a further 48h; * $p < 0.05$ by t test (error bars represent SD).

Fig. 7. Noncanonical Wnt-3a and ATF2 promote neuronal differentiation in iPS-derived neural progenitor cells. (A) Immunofluorescence staining for DCX in differentiating cells treated for 5 days with vehicle (DMSO and CHAPS), Wnt-C59 (100 nM) and vehicle (CHAPS), Wnt-C59 (100 nM) and recombinant Wnt-3a (100 ng/ml), and Wnt-C59 (100 nM), recombinant Wnt-3a (100 ng/ml) and IWR (10 μ M); DAPI staining of nuclei is in blue; scale bars 200 μ m. (B) Relative quantification of DCX levels; * $p < 0.05$ by t test (error bars represent SEM). (C) mRNA expression determined by q-PCR of the genes indicated, using differentiating cells treated as indicated in (A); * $p < 0.05$ by ANOVA test (error bars represent SD). (D) Immunofluorescence staining for DCX in differentiating cells silenced with control (siC) or ATF2 siRNA for 5 days; DAPI staining of nuclei is in blue; scale bars 200 μ m. (E) Relative quantification of DCX levels; * $p < 0.05$ by t test (error bars represent

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SD). (F) Immunofluorescence staining of P-ATF2 in differentiating cells treated as indicated in (A).

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Bengoa, FIG.1

264x352mm (72 x 72 DPI)

Bengoa, FIG.2

264x352mm (72 x 72 DPI)

Bengoa, FIG.3

264x352mm (72 x 72 DPI)

Bengoa, FIG.4

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Bengoa, FIG.5

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Bengoa, FIG.7

264x352mm (72 x 72 DPI)

Supplemental Information**Supplemental Table 1.** Primers used for q-PCR

Primer	Sequence 5' → 3'	Primer Concentration (nM)
NEUROGENIN2 F	CGCATCAAGAAGACCCGTAG	300
NEUROGENIN2 R	GTGAGTGCCCAGATGTAGTTGTG	300
AXIN2 F	AAGTGCAAACCTTTCGCCAAC	300
AXIN2 R	ACAGGATCGCTCCTCTTGAA	300
ASCL1 N F	GAGCAGGAGCTTCTCGACTT	300
ASCL1 N R	CAACGCCCACTGACAAGAAAG	300
NEURO D1 F	ACGAGGAATTGCCCCACGCA	300
NEURO D1 R	CGCCCATCAGCCCACTCTCG	300
NEUROGENIN1 F	CGACACCAAGCTCACCAAAA	900
NEUROGENIN1 R	GCCAGGCGCAGTGTCTC	900
FZD6 F	TCCCAGATGTATGAAAATGGC	900
FZD6 R	CCAGATTTGCGAGAGGAAGA	900
FZD7 F	CGCCTCTGTTCTGTCTACCTC	300
FZD7 R	GTCGTGTTTCATGATGGTGC	300
WNT7A F	CCGGACTCTCATGAACTTGC	900
WNT7A R	GAAACTGTGGCAGTGTGGTC	900
WNT3A F	GTGGAAGTGCACCACCGT	900
WNT3A R	ATGAGCGTGTCACTGCAAAG	900
DKK1 F	ATGCGTCACGCTATGTGCT	900

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DKK1 R	TCTGGAATACCCATCCAAGG	900
WNT7B F	TGGCGTCCTGTACGTGAAGCTC	900
WNT7B R	CGGGGCTAGGCCAGGAATCTT	900
36B4 F	GTGTTTCGACAATGGCAGCAT	300
36B4 R	AGACACTGGCAACATTGCGGA	300
WNT5B F	GGCTCAGCTTCTGACAGACGCCA	900
WNT5B R	ACATCTCGGGTCTCTGCACCGG	900
WNT5A F	GCTCGCTCGGGTGGCGACTTC	900
WNT5A R	CTTCCAGCCATCCCCAAAGCAACT	900
MYC F	CACCGAGTCGTAGTCGAGGT	900
MYC R	TTTCGGGTAGTGGAACCA	900
NKD1 F	ACTTCCAGCCGAAAGTCGT	900
NKD1 R	CACCATAGGCCGAAGCAC	900
WNT4 F	AAGGCCATCCTGACACACATG	300
WNT4 R	GCTAGGCTCCAAGTACACCAGG	300
DCX F	AAGGACCTGTACCTGCCTCT	300
DCX R	TGAGCACTCTCCCCTCCTTT	300
PAX6 F	AGCCCCATATTCGAGCCCCGT	300
PAX6 R	TGGCCGCCCCGTTGACAAAGA	300
CD44 F	CAAGTTTTGGTGGCACGCAG	300
CD44 R	GTGGAATACACCTGCAAAGCG	300
S100B F	GGGAGACAAGCACAAGCTGAA	300
S100B R	CATTCGCCGTCTCCATCATTG	300
OLIG1 F	TACTATGCGGTTTCCCAGGC	300

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OLIG1 R	ACCAGCTCGTAGAGGGAGG	300
OLIG2 F	CCCCTAAAGGTGCGGATGC	300
OLIG2 R	CTTTCGCAGGGTCCTCCTTC	300
PDGFRA F	GGAGGAGAAGTTTCCCAGAGC	300
PDGFRA R	AGGGTAATGAAAGCTGGCAGA	300

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Supplemental Table 2. Antibodies used for immunofluorescence

Antibody	Reference	Species	Dilution
NESTIN	Santa Cruz sc-23927	Mouse	1:200
NANOG	R&D AF1997	Goat	1:15
SOX2	Santa Cruz sc-17320	Goat	1:25
NEUROD1	Santa Cruz sc-46684	Mouse	1:50
βIII TUBULIN	Millipore MAB1637	Mouse	1:50
ATF2	Santa Cruz sc-6233	Rabbit	1:50
P-ATF2	Cell Signaling #5112	Rabbit	1:50
P-ATF2	Santa Cruz sc-7982-R	Rabbit	1:200
cJUN	Cell Signaling #9165	Rabbit	1:25
β-CATENIN	Homemade	Rabbit	1:100
P-CREB	Cell Signaling #9198	Rabbit	1:50
DCX	Santa Cruz sc-8066	Goat	1:100
PAX6	Santa Cruz sc-81649	Mouse	1:100

Supplemental Table 3. Antibodies used for western blotting

Antibody	Reference	Species	Dilution
PAX6	Santa Cruz sc-81649	Mouse	1:1000
NEUROD1	Santa Cruz sc-46684	Mouse	1:1000
βIII TUBULIN	Millipore MAB1637	Mouse	1:2000
HSP60	Santa Cruz sc-13966	Rabbit	1:2000
GAPDH	Sigma G8795	Mouse	1:2000
cJUN	Cell Signaling #9165	Rabbit	1:1000
ATF2	Santa Cruz sc-6233	Rabbit	1:1000
P-ATF2	Santa Cruz sc-7982-R	Rabbit	1:1000
P-CREB	Cell Signaling #9198	Rabbit	1:1000
βCAT	BD610154	Mouse	1:2000
LAM A	Genscript A01455	Rabbit	1:1000

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Legends for supplemental online videos

Video 1. *Wnt7b* mRNA expression in the mouse brain ISH data from the Allen Mouse Brain Atlas [50] were visualized using Brain Explorer® software (<http://mouse.brain-map.org/static/brainexplorer>). The top left corner legend indicates which areas of the mouse brain are being shown, the top right inset is a guide for the actual position of the mouse brain and the bottom right legend is an indicator of the intensity and density of the staining.

Video 2. *ATF2* mRNA expression in the mouse brain ISH data from the Allen Mouse Brain Atlas [50] were visualized using Brain Explorer® software (<http://mouse.brain-map.org/static/brainexplorer>). The top left corner legend indicates which areas of the mouse brain are being shown, the top right inset is a guide for the actual position of the mouse brain and the bottom right legend is an indicator of the intensity and density of the staining.

Supplemental Figure Legends**Fig. S1. Characterization of hNP cells**

(A-E) Immunofluorescence staining of hNP cells to detect NESTIN (NES, green (A), SOX2 (green) (B), NANOG (red) & β III-tubulin (β IIIT, green) (C), NEUROD1 (ND1, green) (D) and β -catenin (β CAT, red) (E) in undifferentiated (UNDIF) cells and after 3 days of differentiation (DIF); DAPI staining of nuclei is in blue; scale bar 100 μ m. (F) Western blots detecting NEUROD1 (ND1), PAX6 and β III-tubulin (β IIIT) in total extracts of hNP cells during 5 days of differentiation; HSP60 was used as a loading control.

Fig. S2. Analysis of changes in Wnt pathway gene expression during hNP cell differentiation using Taqman® arrays

(A) mRNA expression of the neuronal genes *NEUROD1* (ND1) and *ASCL1* in RNA from undifferentiated (UNDIF) or 3 day-differentiated (DIF) hNP cells, as used for analysis in the Taqman® array. (B) Top hits, as defined by fold-change in signal using the Taqman® Human WNT Pathway array, with genes downregulated in differentiating cells in green and upregulated genes in red. (C-D) Graphs show average mRNA expression determined by q-PCR of the genes indicated in undifferentiated (UN) or differentiating (DIF) cells and cells treated with DMSO or Wnt-C59 100 nM. (E) mRNA expression determined by q-PCR of the genes indicated in hNP cells treated with vehicle (DMSO and/or CHAPS), Wnt-5a (100 ng/ml) and/or IWP-2 for 72 h; * $p < 0,05$ by ANOVA test (error bars represent SD).

Fig. S3. Dkk-1 blocks Wnt/ β -catenin signaling.

(A) Cartoon illustrating Dkk-1 inhibition of canonical but not noncanonical Wnt signaling. (B) Gene reporter assays measuring β -catenin/Tcf activity (TOPFlash/FOPFlash ratio) in hNP

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cells treated with vehicle (CHAPS), Wnt-3a (100 ng/ml) and/or Dkk-1 (100 ng/ml) for 24 h; *
 $p < 0,05$ by ANOVA (error bars represent SD).

Fig. S4. Noncanonical Wnt-3a increases phosphorylation and expression of AP-1 family members.

(A-D) Immunofluorescence staining for phospho-CREB (A), phospho-ATF2 (B), ATF2 (C) and c-Jun (D) in differentiating hNP cells treated Wnt-C59 (100 nM) and vehicle (CHAPS), recombinant Wnt-3a (W3A, 100 ng/ml) and recombinant Dkk-1 (100 ng/ml) or both together; DAPI staining of nuclei is in blue; scale bars 200 μ m.

Fig. S5. Wnt-3a regulation of AP-1 is unaffected by Dkk-1 and reduced by the JNK inhibitor JI8.

(A) Gene reporter assays measuring AP-1 activity in hNP cells treated with vehicle (DMSO) or JI8 (10 μ M). (B) Quantification of the 3 independent WB experiments analyzed with ImageJ. (C) Graphs show average mRNA expression determined by q-PCR of the genes indicated, relative to each control siRNA sample; * $p < 0.05$ by t test (error bars represent SD).

Fig. S6. Characterization of iPS-derived neural progenitor cells

(A-C) Immunofluorescence staining of iPS-derived neural progenitor cells to detect DCX (A), NESTIN (green) and SOX2 (red) (B), and PAX6 (green) and P-ATF2 (red) (C) at 5 days of differentiation; DAPI staining of nuclei is in blue; scale bar 200 μ m. (D) Western blots detecting ND1, PAX6 and DCX in total extracts of iPS-derived neural progenitor cells during 5 days of differentiation; GAPDH was used as a loading control.

Fig. S7. Neuronal and Wnt pathway gene expression during neuronal differentiation of iPS-derived neural progenitor cells

(A-C) Graphs show average mRNA expression determined by q-PCR of the genes indicated, from 3 independent 7-day time-course experiments (error bars represent SEM). (D) q-PCR of the genes indicated, relative to *36B4*; * $p < 0.05$ by *t* test (error bars represent SD). (E) mRNA expression determined by q-PCR of the genes indicated, relative to *36B4*, from 3 experiments using differentiating cells treated for 5 days with vehicle (DMSO and CHAPS), Wnt-C59 (100 nM) and vehicle (CHAPS), Wnt-C59 (100 nM) and recombinant Wnt-3a (100 ng/ml), and Wnt-C59 (100 nM), recombinant Wnt-3a (100 ng/ml) and IWR (10 uM); * $p < 0.05$ by ANOVA test (error bars represent SD).

Fig. S8. Expression of non-canonical Wnt pathway components in mouse dentate gyrus

In situ hybridization of *Wnt7B* (A), *Creb* (B) and *Atf2* (C) mRNAs in the dentate gyrus from from the Allen Mouse Brain Atlas (<http://mouse.brain-map.org> [50]).

Fig. S9. Inhibition of alternative pathways during neuronal differentiation of hNP cells

(A-B) Graphs show average mRNA expression determined by q-PCR of the genes indicated, upon silencing of the indicated genes; * $p < 0.05$ by ANOVA test (error bars represent SD). (C-D) mRNA expression of neuronal genes in differentiating hNP cells treated with Rho kinase inhibitor Y-27632 and the CAMKII inhibitor myr-AIP, $n = 1$.

Bengoa, FIG. S1

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Bengoa, FIG. S9

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