

Dopaminergic neuronal cluster size is determined during early forebrain patterning

Niva Russek-Blum¹, Amos Gutnick¹, Helit Nabel-Rosen¹, Janna Blechman¹, Nicole Staudt², Richard I. Dorsky³, Corinne Houart² and Gil Levkowitz^{1,*}

We have explored the effects of robust neural plate patterning signals, such as canonical Wnt, on the differentiation and configuration of neuronal subtypes in the zebrafish diencephalon at single-cell resolution. Surprisingly, perturbation of Wnt signaling did not have an overall effect on the specification of diencephalic fates, but selectively affected the number of dopaminergic (DA) neurons. We identified the DA progenitor zone in the diencephalic anlage of the neural plate using a two-photon-based uncaging method and showed that the number of non-DA neurons derived from this progenitor zone is not altered by Wnt attenuation. Using birthdating analysis, we determined the timing of the last cell division of DA progenitors and revealed that the change in DA cell number following Wnt inhibition is not due to changes in cell cycle exit kinetics. Conditional inhibition of Wnt and of cell proliferation demonstrated that Wnt restricts the number of DA progenitors during a window of plasticity, which occurs at primary neurogenesis. Finally, we demonstrated that Wnt8b is a modulator of DA cell number that acts through the Fz8a (Fzd8a) receptor and its downstream effector Lef1, and which requires the activity of the Fezl (Fezf2) transcription factor for this process. Our data show that the differential response of distinct neuronal populations to the Wnt signal is not a simple interpretation of their relative anteroposterior position. This study also shows, for the first time, that diencephalic DA population size is modulated inside the neural plate much earlier than expected, concomitant with Wnt-mediated regional patterning events.

KEY WORDS: Cell number, Neuronal specification, Oxytocin, Dopamine, Cell lineage

INTRODUCTION

Neural patterning cues, such as Wnt, provide progenitor cells with distinct positional identity and induce proneural proteins, which in turn initiate neurogenesis (Guillemot, 2007). The Wnt signaling pathway plays a major role in the patterning of the anterior embryonic neuroectoderm, which results in the subdivision of the developing brain into discrete compartments (Glinka et al., 1998; Heisenberg et al., 2001; Houart et al., 2002; Lekven et al., 2001; Mukhopadhyay et al., 2001). Recent studies in zebrafish have demonstrated that the canonical Wnt signal performs multiple functions in the anterior neuroectoderm. It acts as a posteriorizing signal, controlling the fates of the posterior diencephalon and mesencephalon (Kim et al., 2002). It interacts with other, non-canonical Wnt signals to induce eye-field formation (Cavodeassi et al., 2005). It acts upstream of Lef1 to positively regulate neurogenesis in the posterior hypothalamus (part of the diencephalon) (Lee et al., 2006). Finally, it has been shown that canonical Wnt signaling is important for the acquisition of hypothalamic versus floor plate identity (Kapsimali et al., 2004). Gain-of-function studies in *Xenopus* and chick further demonstrate that graded activation of Wnt signaling induces the expression of defined sets of transcription factors that delineate the various brain regions (Kiecker and Niehrs, 2001; Nordstrom et al., 2002). Despite this wealth of information, the exact contribution of Wnt-mediated neural plate patterning to neuronal fate determination remains unclear, particularly regarding the differentiation phenotypes of distinct neuronal subtypes in the forebrain.

The development of diencephalic dopaminergic (DA) neurons may serve as a paradigm for how robust patterning signals contribute to diencephalic subtype specification. Diencephalic DA cells are present in the hypothalamus, ventral thalamus and caudal diencephalon (Smeets and Reiner, 1994). In mammals, these neurons are essential for a variety of vital neural functions, including regulation of sympathetic preganglionic neurons and hormone secretion, as well as for control of motivational responses and sexual arousal (Dominguez and Hull, 2005; Iversen et al., 2000; Paredes and Agmo, 2004). In the mesencephalon, the development of DA neurons is positively regulated by Wnt signaling, *in vitro* (Castelo-Branco et al., 2003; Schulte et al., 2005) and *in vivo* (Prakash et al., 2006). However, the role of Wnt signaling in diencephalic DA neural development has not been studied. We and others have investigated the embryonic development of DA neurons in the zebrafish (Blechman et al., 2007; Guo et al., 1999; Holzschuh et al., 2003; Levkowitz et al., 2003; Ryu et al., 2007). Unlike in mouse embryos, the small DA neuron population size in the zebrafish diencephalon enables accurate quantitative analysis of even small changes in the number of differentiated neurons.

A crucial question addressed herein is whether forebrain-patterning cues, such as the canonical Wnt signal, affect the specification and number of distinct diencephalic neuronal populations. We show that in the zebrafish embryo, the number of diencephalic DA cells is almost invariant and is subject to tight control by the Wnt/ β -catenin pathway. Our findings show that Wnt-mediated signaling does not have an overall effect on the differentiation of neuronal subtypes in the diencephalon. Instead, Wnt signaling limits the initial pool of DA progenitors at the neural plate stage, during a window of plasticity that coincides with the onset of primary neurogenesis, earlier than previously described (Rink and Wullmann, 2002; Smidt and Burbach, 2007). We suggest that the control of neuronal cluster size by early patterning signals plays a crucial role in delineating the upper and lower size limits of selected diencephalic neuronal clusters.

¹Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel. ²Medical Research Council Centre for Developmental Neurobiology, King's College, London SE1 1UL, UK. ³Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132, USA.

*Author for correspondence (e-mail: gil.levkowitz@weizmann.ac.il)

MATERIALS AND METHODS

Plasmids, probes and fish lines

Full-length *dkk1* cDNA was amplified by RT-PCR of RNA isolated from zebrafish embryos at 9 hours post-fertilization (hpf) and the cDNA was subcloned into either the pCS2+ expression vector or a Tol2 transposon-based plasmid (Kawakami, 2004) containing a heat-shock 70/4 promoter (Halloran et al., 2000). Sequences of primers used to amplify DNA templates for preparation of digoxigenin (DIG)- or fluorescein (FITC)-labeled probes are available upon request. The *fezl* and *barhl2* probes have been described previously (Colombo et al., 2006; Levkowitz et al., 2003). The *tof*^{m808} mutant, *X8* mutant, Tg(1.4*dlx5a-dlx6a:gfp*) and Tg(-8.4*neurog1:gfp*) lines have been described previously (Blader et al., 2003; Ghanem et al., 2003; Lee et al., 2006; Levkowitz et al., 2003).

Morpholino and mRNA injections

Antisense morpholino oligonucleotides (MOs) (Gene Tools) targeted to the translation start site of *fz8a* or *wnt8b* and to an RNA-splicing site of *lef1* are as previously described (Houart et al., 2002; Kim et al., 2002; Lee et al., 2006). *dkk1* mRNA (20 pg/embryo), *fz8a* (5 ng/embryo), *wnt8b* (3 ng/embryo) and *lef1* (2 ng/embryo) MOs were injected at the 1-cell stage and embryos allowed to develop at 28.5°C.

Quantification of cell number

Cell numbers in confocal images were quantified using ImageJ. Each channel was analyzed separately and converted to grayscale to maximize contrast. Using the Point Selection tool, the center of each cell was marked with a blocked circle (seven pixels in diameter) and its *x*, *y*, *z* position was automatically logged. Frequent cycling between adjacent *z*-planes ensured that no cell was logged more than once. Volocity software (Improvision, Covington, UK) was used to corroborate these cell counts and to quantify other large control domains. The software identified as an individual cell any object with a calculated volume between 100 μm^3 and 150 μm^3 and signal intensity no lower than 20% of the normalized distribution of signal intensities in the entire image.

Fate mapping

High-resolution fate mapping of diencephalic progenitor zones was performed by a two-photon-based photo uncaging procedure using a Zeiss LSM 510 microscope equipped with a 720-nm Mai Tai laser. We used the *neurog1::gfp* reporter line [Tg(-8.4*neurog1:gfp*)] as a live genetic landmark of the diencephalic territory. *neurog1::gfp* transgenic embryos were injected with 1% DMNB-caged fluorescein in 0.2 M KCl immediately after fertilization, and were then grown in the dark. Embryos were mounted at the bud stage in 1% low-melting-point agarose in E2 medium. The caged fluorescein was converted into its fluorescent, active form at discrete diencephalic domains using the Region of Interest (ROI) function (720 nm, 20 iterations at a scan speed of 25.6 $\mu\text{sec}/\text{pixel}$). The uncaged fluorescein and DA neurons were visualized at the prim5 stage (24 hpf) using an alkaline phosphatase-coupled anti-fluorescein antibody followed by Fast Red staining. The embryos were then stained with anti-tyrosine hydroxylase antibody to visualize DA neurons.

Cell-proliferation analysis

BrdU labeling (10 mM BrdU in E2 medium) was conducted as described (Shepard et al., 2004). To block cell division, embryos were dechorionated and incubated in a combination of 1 $\mu\text{g}/\text{ml}$ aphidicolin and 50 mM 5-hydroxyurea in 4% DMSO. Control embryos were incubated in 4% DMSO. Cell cycle inhibitors were removed at 24 hpf by washing three times with embryo medium (E3).

RESULTS

The Wnt antagonist Dkk1 affects DA cell number but does not impair their differentiation

Gain-of-function mutations of canonical Wnt antagonists result in enlarged heads and promote telencephalic neural fates in both *Xenopus* and zebrafish embryos (Glinka et al., 1998; Hashimoto et al., 2000a; Houart et al., 2002; Shinya et al., 2000). However, the effect of this forebrain mispatterning on the specification and

number of distinct diencephalic neuronal populations has not been addressed. Members of the canonical Wnt family are necessary for precursor proliferation, fate decisions and neuronal differentiation of midbrain DA neurons (reviewed by Prakash and Wurst, 2006; Smidt and Burbach, 2007). We therefore examined whether perturbation of anteroposterior patterning by ectopic expression of the canonical Wnt antagonist dickkopf 1 (*Dkk1*), might similarly impair the development of diencephalic DA neurons.

In zebrafish, DA groups develop in a ventral diencephalic area termed the posterior tuberculum (PT) (Holzschuh et al., 2001; Rink and Wullmann, 2002). Within this domain, distinct DA clusters can be readily identified in the 1- to 5-day-old zebrafish embryo (Fig. 1A) (Rink and Wullmann, 2002). We analyzed the effects of *Dkk1* on the two major diencephalic DA clusters, group 2 and groups 3-6, using a transgenic line expressing GFP under the control of the *dlx6a* promoter (Ghanem et al., 2003). These transgenic embryos (denoted *dlx6a::gfp*) express GFP in the telencephalon and in two discrete domains of the diencephalon (Fig. 1C,E,I). An anterior expression domain, located just posterior to the telencephalon-diencephalon boundary, demarcates the proliferating zone that gives rise to the group 2 DA cluster, which appears 22-24 hours post-fertilization (hpf) (data not shown), and an expression domain perpendicular to this boundary demarcates a proliferating zone contiguous with group 3-6 DA neurons, which appear at 30-48 hpf (Fig. 1C,E,I).

Surprisingly, when mRNA encoding the *Dkk1* protein was injected into zebrafish embryos, we were still able to detect all known neuronal clusters of diencephalic DA cells (Fig. 1B,D,F). We then took advantage of the small and virtually invariant cell population size in the zebrafish diencephalon to examine whether DA cell number is altered following attenuation of canonical Wnt signaling. Quantitative analysis revealed that *dkk1*-injected embryos had a two-fold increase in tyrosine hydroxylase (TH)-positive group 2 DA neurons and a moderate increase in the number of group 3-6 DA cells (Figs 1B,D,F and see Fig. S1 in the supplementary material). Similarly, a two-fold increase in dopamine transporter (*dat*; *slc6a3*)-positive neurons was observed, starting from the earliest stage in which differentiated group 2 DA neurons are detected (22 hpf) and persisting through 5 days of development (Fig. 1G and data not shown). In contrast to DA neurons, the number of *Dlx6a*⁺ cells in the basal diencephalon was unaltered by Wnt attenuation, suggesting that not all ventral diencephalic progenitors are affected by Wnt perturbation (Fig. 1E,F,H). Thus, attenuation of the canonical Wnt signal by *Dkk1* specifically alters the number of diencephalic DA neurons.

Wnt attenuation specifically elevates DA cell number without affecting the progeny of adjacent progenitor populations

Dkk1 might have an overall effect on the specification of diencephalic fates, including DA neurons. We therefore examined whether *Dkk1* affects terminal differentiation as well as cell number of other diencephalic cell types that develop in juxtaposition to DA neurons. We observed that oxytocinergic neurons [termed isotocinergic (IT) in fish] develop just anterior to DA group 2, whereas hypocretin (Hcrt)-secreting neurons are formed in a slightly ventral position (Fig. 2A,C,G). Somatostatin (SS)-producing cells appear in several clusters located in the ventral (preoptic area) and dorsal (thalamus) diencephalon (Fig. 2E,G) (Blechman et al., 2007). Simultaneous examination of DA neurons, together with each of the other cell types, revealed that ectopic expression of *Dkk1* had no effect on the cellular organization and number of either IT, Hcrt or SS producing neurons (Fig. 2B,D,F,H). These results suggest that although

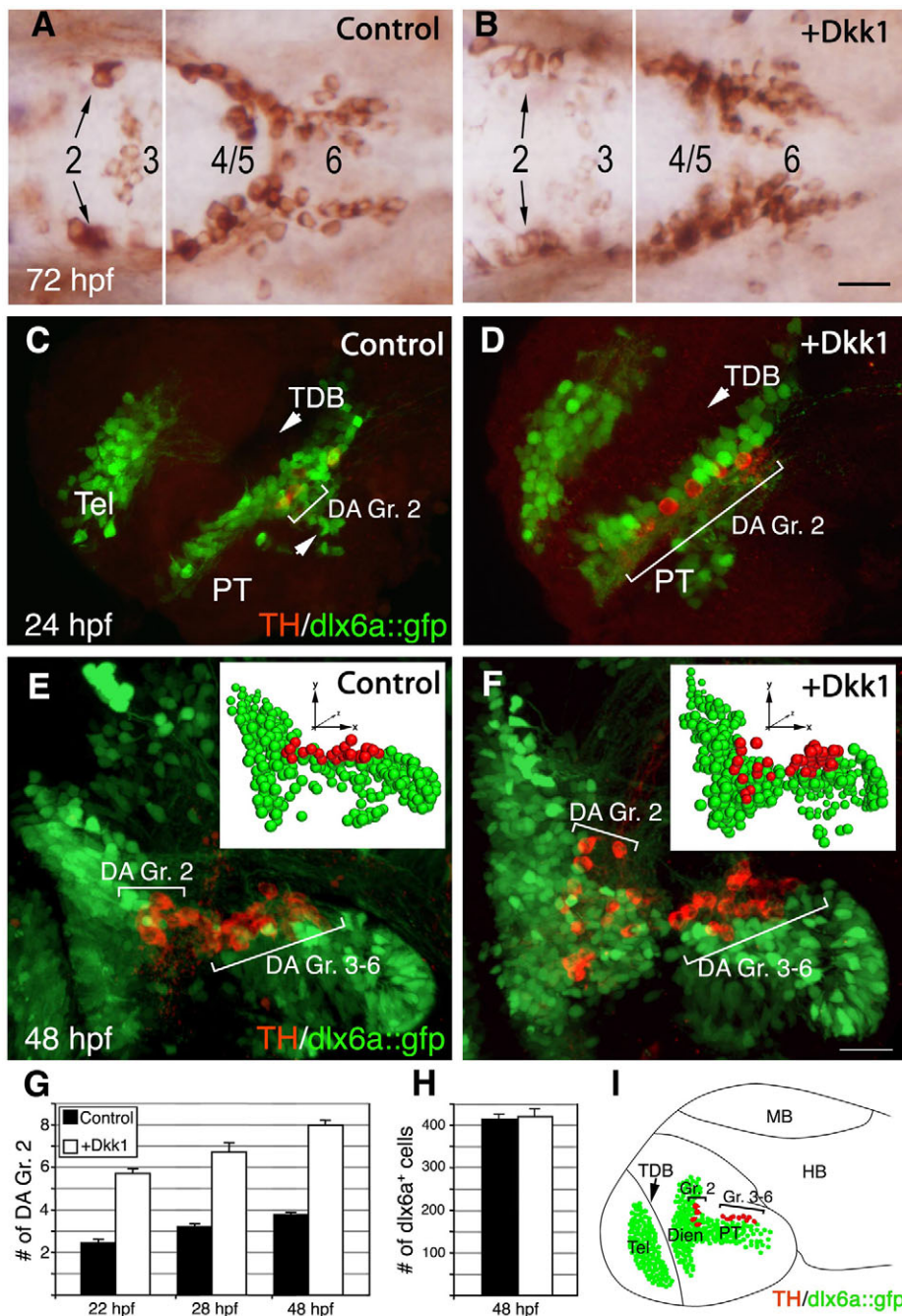


Fig. 1. The Wnt antagonist Dkk1 causes an increase in DA cell number.

(A,B) Dorsal views of zebrafish embryos injected with (A) vehicle solution or (B) an in vitro synthesized capped mRNA encoding Dkk1. Embryos were fixed at 72 hours post-fertilization (hpf) and subjected to immunostaining with an anti-tyrosine hydroxylase (TH) antibody to detect dopaminergic (DA) neurons. Diencephalic DA groups [as annotated by Rink and Wullmann (Rink and Wullmann, 2002)] are marked. (C-F) Projected confocal z-stack images of DA neurons (red) in transgenic embryos expressing GFP under the control of the *dlx6a* promoter (*dlx6a::gfp*; lateral view). Embryos were injected with either a vehicle solution (C,E) or *dkk1* mRNA (D,F), and were fixed at either 24 (C,D) or 48 (E,F) hpf. The insets in E and F show bubble-plot graphic representations of the x, y, z cell position of all *dlx6a*⁺ (green) and TH⁺ (red) cells in the ventral diencephalon. Bubble size was derived from the position of the cells along the z-axis to give the impression of depth. The positions of DA group 2 (Gr. 2) and groups 3-6 (Gr. 3-6) are indicated. (G) Bar chart showing average counts of dopamine transporter (Dat)-expressing DA group 2 (Gr. 2) cells in WT (control) and *dkk1*-injected embryos. Dat⁺ neurons were detected by whole-mount in situ hybridization with *dat* probe at 22, 28 and 48 hours post-injection ($n=25$). (H) Bar chart showing average counts of *dlx6a*⁺ cells in the basal diencephalon of WT and *dkk1*-injected *dlx6a::gfp* transgenic embryos ($n=5$). (I) Schematic depicting the major diencephalic DA cell groups that appear in the developing embryo at 48 hpf, relative to the *dlx6a::gfp* expression domain. Dien, diencephalon; HB, hindbrain; MB, midbrain; PT, posterior tuberculum; Tel, telencephalon; TDB, telencephalic-diencephalic boundary. Scale bars: 25 μ m.

inhibition of canonical Wnt signaling affects forebrain patterning, it does not affect the fate and number of all diencephalic cell populations.

To further distinguish patterning signals generally affecting the size of diencephalic territories from changes in specific neuronal precursor pools, we set out to identify the primordial diencephalic area from which DA precursors are derived. A recent fate-mapping analysis of the diencephalon demonstrated that diencephalic progeny of discrete gene expression domains of the neural plate develop as a continuous clone, and do not display cell mixing with neighboring regions (Staudt and Houart, 2007). As Staudt and Houart showed that some gene expression domains in the neural plate seem to be retained by the same cells up to the prim5 stage (24 hpf), we sought genetic landmarks expressed in the diencephalic anlage at bud stage (end of gastrulation), as well as in the PT of

embryos at 24 hpf. Thus, triple staining of the pro-neural marker *neurogenin 1* (*neurog1*), the diencephalic marker *BarH-like 2* (*barhl2*) and TH in 24 hpf embryos showed that the *neurog1*⁺ group 2 DA cluster appeared adjacent to the *barhl2*⁺ PT domain (see Fig. S2 in the supplementary material).

Based on these gene expression data, we hypothesized that DA progenitors originate in or around this area. We then performed fate-mapping analyses using the *neurog1::gfp* transgenic line (Blader et al., 2003), which served as a live landmark for high-resolution visualization of the *neurog1*⁺ cells in the diencephalic anlage during neurulation (Fig. 3A). To carry out high-resolution fate mapping of diencephalic progenitors, we developed a new photo-activation (i.e. uncaging) strategy using two-photon microscopy, which enables activation of fluorescein-conjugated tracer dye at highly localized focal points. We precisely activated the fluorescein tracer in a small

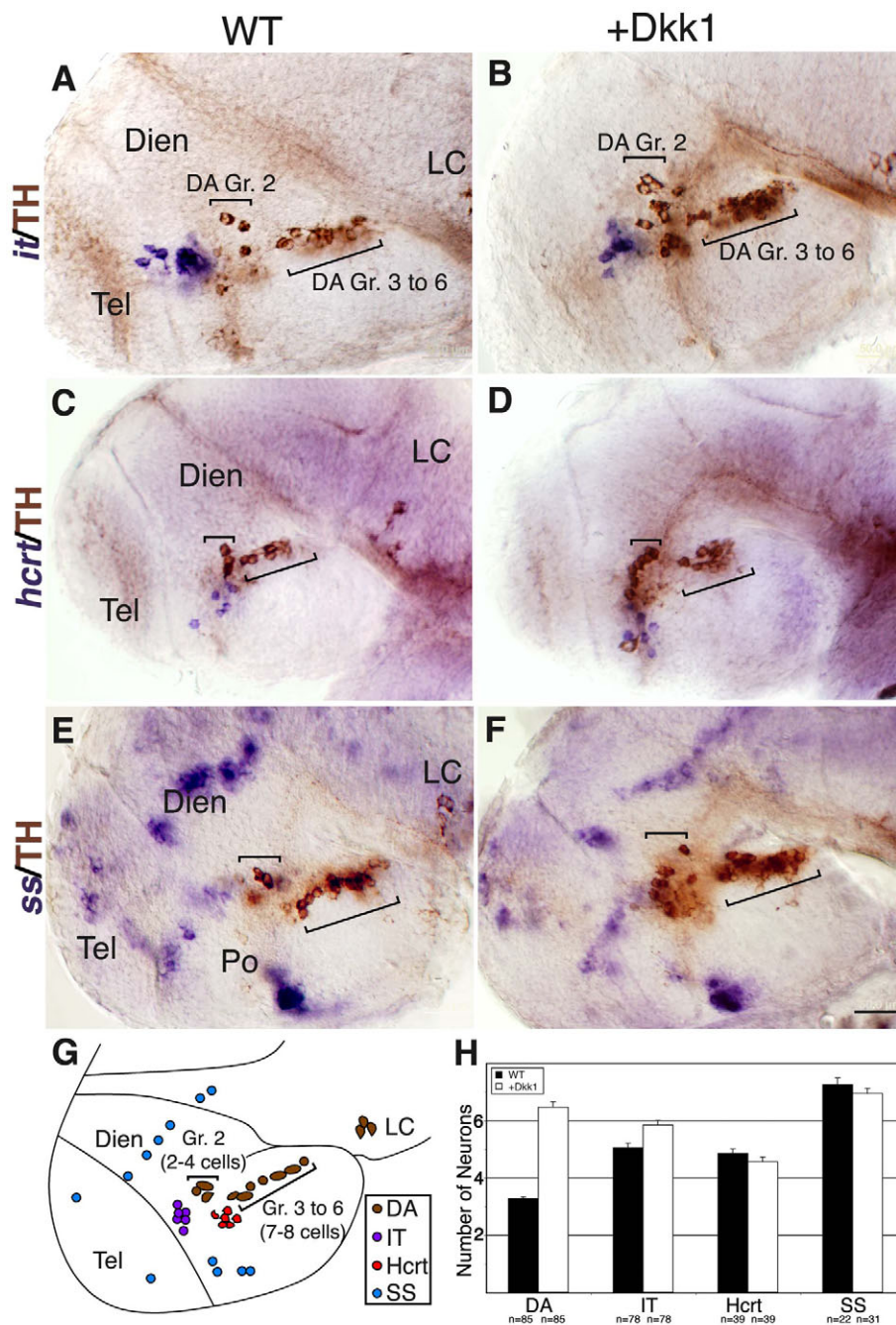


Fig. 2. *Dkk1* selectively affects DA cell number, but not neighboring basal diencephalic neurons. (A-F) Lateral views of either WT zebrafish embryos (A,C,E) or their *dkk1* mRNA-injected siblings (B,D,F). Embryos were fixed at 48 hpf and subjected to whole-mount in situ hybridization with RNA probes directed against the hypothalamic neuropeptide isotocin (zebrafish ortholog of oxytocin) (*it*; A,B), hypocretin (*hcrt*; C,D) or somatostatin (*ss*; E,F). All specimens were then subjected to immunostaining with an anti-tyrosine hydroxylase (TH) antibody to detect DA neurons. (G) Schematic representation of the examined diencephalic cell types in a 48-hpf WT embryo. The positions of DA group 2 (Gr. 2) and groups 3-6 (Gr. 3 to 6) are indicated. (H) Bar chart presenting average counts of *dat*-expressing DA group 2 cells (DA), isotocin (IT), hypocretin/orexin (Hcrt) or somatostatin (SS) expressing cells in WT and *dkk1*-injected embryos at 48 hpf. The number of embryos analyzed (n) is shown below. Dien, diencephalon; LC, locus coeruleus; Po, preoptic area; Tel, telencephalon. Scale bar: 50 μ m.

group of cells at the basal diencephalic anlage of the *neurog1::gfp* transgenic embryos at the 1- to 3-somite stage (Fig. 3A and see Fig. S3 in the supplementary material). We then allowed the embryos to develop until 24 hpf, at which time terminally differentiated DA neurons are readily detectable (Fig. 3B,C). Using this method, we located three discrete diencephalic progenitor pools; one of these, denoted domain II, reproducibly labeled *Neurog1*⁺ TH⁺ DA neurons in both wild-type (WT) and reduced Wnt conditions (Fig. 3A-D, *n*=6, and see Fig. S3 in the supplementary material). Furthermore, uncaging other diencephalic domains around domains I and II in *Dkk1*-overexpressing embryos did not label DA cells, indicating that DA progenitors remain in the same neural plate position under reduced Wnt conditions (data not shown).

The uncaged DA cells in both WT and *dkk1*-injected embryos express TH and the *neurog1* tracer (Fig. 3 and see Fig. S2 in the supplementary material). We used the above mapping information to quantify the effect of the reduced Wnt conditions on *neurog1*⁺ progenitor pools at a later embryonic stage. Strikingly, we found that within the PT, the number of TH⁺ *neurog1*⁺ DA cells was markedly affected by overexpression of *Dkk1*, whereas the total number of *neurog1*⁺ cells in this domain was not significantly altered (Fig. 4). Given our finding that PT progenitors, including DA neurons, originate from the same position within the neural plate, we conclude that specific *neurog1*⁺ precursor pools respond differentially to Wnt signaling.

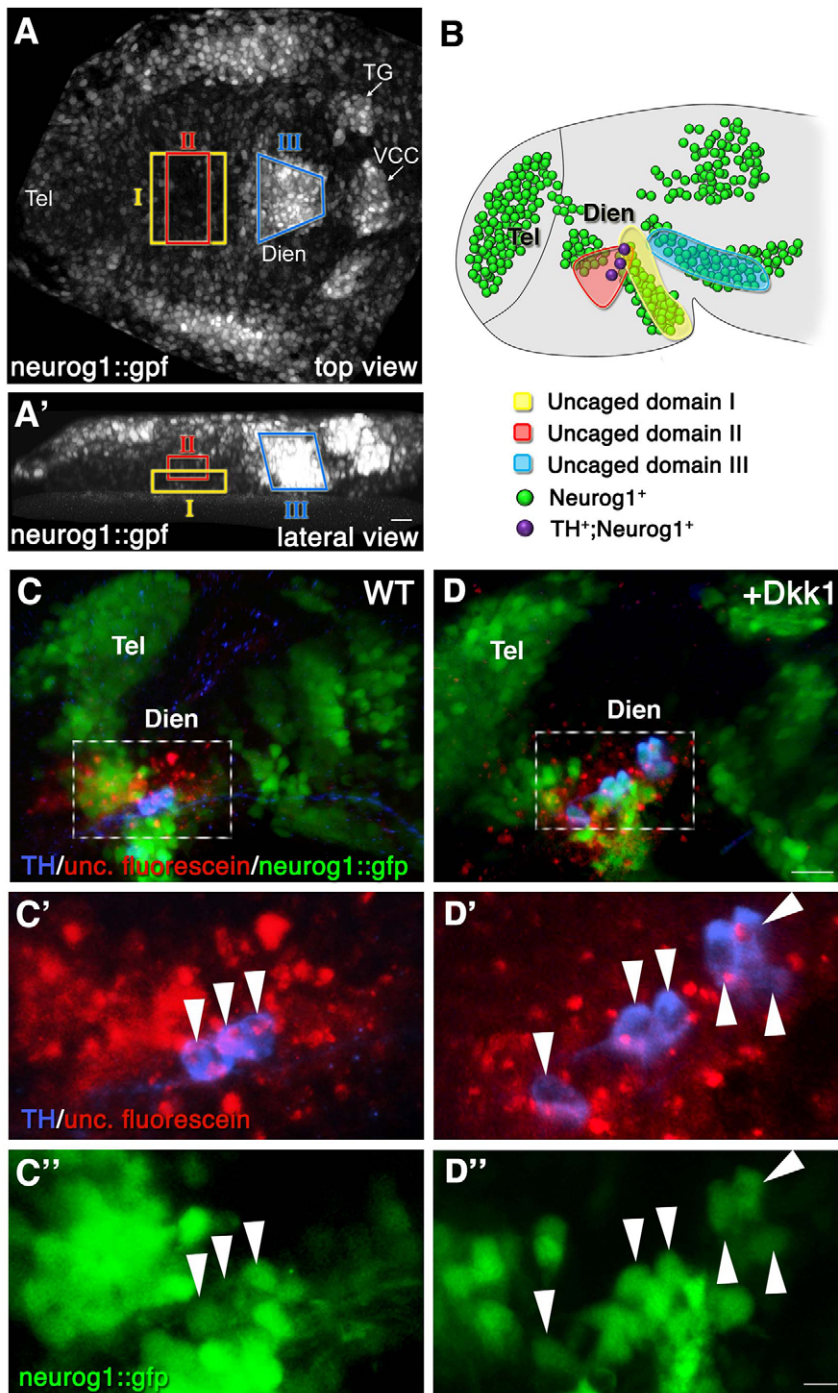


Fig. 3. Fate mapping of the diencephalic progenitor zones. High-resolution fate mapping by two-photon-based uncaging procedure. **(A,A')** Zebrafish embryos (anterior to the left) expressing GFP under the control of the *neurog1* promoter (*neurog1::gfp*) were injected with caged dextran-fluorescein tracer dye at the 1-cell stage. At the 1- to 3-somite stage, the dye was uncaged at the indicated domains (denoted I, II and III) of the diencephalic anlage. **(A)** Dorsal view; **(A')** lateral view. **(B)** Schematic summarizing the results of multiple uncaging experiments showing the final destination of the fluorescein-labeled cells in 24-hpf *neurog1::gfp* embryos. The clones corresponding to each of the uncaged domains are color coded (I, $n=6$; II, $n=6$; III, $n=10$). **(C-D')** Control (WT; C-C'') and *dkk1* mRNA-injected (D-D'') embryos that underwent uncaging were fixed at 24 hpf, followed by immunofluorescence staining of the uncaged fluorescein and of TH⁺ DA neurons. High-magnification images of a diencephalic area (dashed boxes in C,D) containing *neurog1*⁺ TH⁺ fluorescein⁺ triple-positive cells (arrowheads) are shown in C', C'', D', D''. Dien, diencephalon; Tel, telencephalon; TG, trigeminal ganglion; VCC, ventrocaudal cluster. Scale bars: 25 μ m in A-D; 50 μ m in C', C'', D', D''.

Cellular responses underlying Wnt-mediated restriction of DA cell number

The identity and number of progenitor cells may be influenced by local instructive cues affecting the progenitor zone and by transcription factors determining the timing of the last cell division of the progenitors (Guillemot, 2007). We next wished to elucidate the type(s) of cellular behavior that leads to the changes in DA cell number in response to Wnt attenuation.

Studies by ourselves and others have ruled out the possibility that cell survival/apoptosis play a major role in the neurogenesis of the basal diencephalon, including the DA neurons (Furutani-Seiki et al., 1996; Guo et al., 1999; Jeong et al., 2006; Lee et al., 2006; Rink and

Guo, 2004). The supernumerary DA cell phenotype reported herein might occur because DA progenitors undergo an extra cell division. However, the exact developmental stage at which zebrafish DA neurons exit the cell cycle (termed birthdate) is not known. We therefore performed birthdating analysis on WT and *dkk1*-injected embryos. Embryos were labeled with 5-bromo-2'-deoxyuridine (BrdU) at different developmental time points, and DA neurons were immunostained subsequent to their terminal differentiation (Fig. 5 and see Table S1 in the supplementary material). We then analyzed the proportion of proliferating DA progenitors by double immunofluorescence staining of progenitors with anti-BrdU (nuclear staining) and of differentiated DA cells with anti-TH

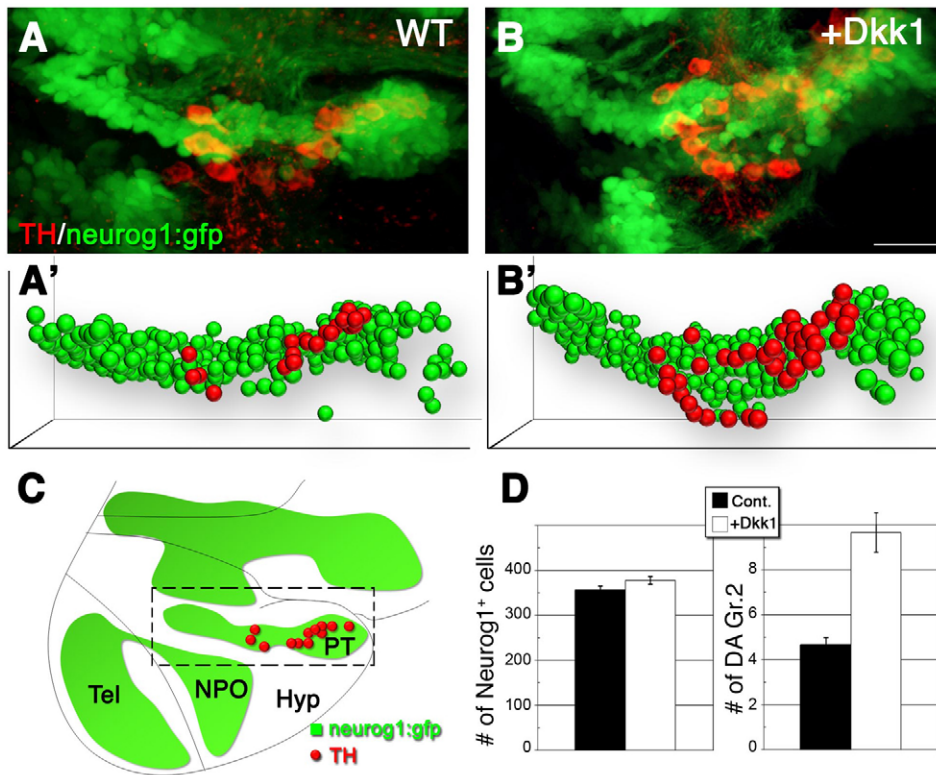


Fig. 4. Specific *neurog1*⁺ precursor pools differentially respond to Wnt signaling. (A,B) Projected confocal z-stack images (lateral view) of DA (red) and *neurog1*⁺ (green) cells in *neurog1::gfp* transgenic zebrafish reporter embryos. Embryos were injected with either a vehicle solution (A) or an mRNA encoding Dkk1 (B). (A',B') Bubble-plot graphic representation of the x, y, z cell position of all *neurog1*⁺ (green) and DA (TH⁺ *neurog1*⁺) cells (red) in the PT. Bubble size was derived from the position of the cells along the z-axis to give the impression of depth. (C) Schematic depicting the major diencephalic DA cell groups in the developing embryo at 48 hpf, relative to the *neurog1::gfp* expression domains. The area indicated by the dashed box corresponds to the posterior tuberculum domain as shown at high-magnification in A,B. (D) Bar charts showing the averaged number of *neurog1*⁺ and DA (TH⁺ *neurog1*⁺) cells in embryos that were injected with either a vehicle solution or *dkk1* mRNA (*n*=5). Hyp, hypothalamus; NPO, neurosecretory preoptic nucleus; PT, posterior tuberculum; Tel, telencephalon. Scale bar: 25 μ m.

(cytoplasmic staining) antibodies. This analysis revealed that the first DA cells became postmitotic as early as 10 hpf (bud/1-somite stage). By 16 hours (14- to 15-somite stage) and thereafter, the DA progenitors that gave rise to the TH⁺ neurons detectable at 48 hpf did not incorporate BrdU (Fig. 5A,C,E,G). In accordance with the developmental stage at which terminally differentiated DA neurons appear in the diencephalon, we found that the group 2 DA cluster exits the cell cycle earlier than the group 3-6 cluster (Fig. 5G). Surprisingly, birthdating analysis of DA progenitors in *dkk1*-injected embryos showed that these neurons exhibited identical cell cycle withdrawal kinetics to their WT siblings (Fig. 5B,D,F,G). Furthermore, WT and *dkk1*-injected embryos had the same proportion of cycling DA progenitors at each of the sampled developmental stages (Fig. 5G). Thus, DA progenitors become postmitotic between 10 and 16 hpf, and the expansion of the DA cell population following Wnt inhibition is not due to delayed cell cycle exit of DA progenitors.

An alternative explanation for this increase in DA neurons is that attenuation of Wnt signaling induces a higher rate of progenitor proliferation owing to the shortening of cell cycles. If this were the case, then cell division blockers should mitigate the effect of Dkk1 on DA cell number. To investigate this possibility, we allowed WT and *dkk1*-injected embryos to develop in the presence of the DNA synthesis inhibitors aphidicolin and 5-hydroxyurea (Fig. 6). Under these experimental conditions, cell division in the diencephalon was partially blocked, allowing gross morphogenesis to proceed while blocking proliferation of diencephalic DA progenitors (see Fig. S4 in the supplementary material). We applied the inhibitor cocktail to embryos at early gastrulation (6 hpf), end of gastrulation to onset of neurogenesis (10 hpf) and mid-somitogenesis (14 hpf); these time points correspond to the respective stages of development at which 100, 60 and ~20% of DA progenitors are in a state of proliferation (Fig. 5G and Fig. 6H). Consistent with our birthdating analyses,

blocking cell division at 6 hours had the greatest effect on DA cell number, whereas application of the cell cycle inhibitors at 14 hours had a minimal effect on DA cell development (Fig. 6A,C,E,G). A comparison between WT and *dkk1*-injected embryos showed that the number of DA neurons was doubled in *dkk1*-injected embryos treated with cell division blockers at 6, 10 or 14 hours of development (Fig. 6B,D,F,G). Notably, Dkk1 gain-of-function was able to recover the reduction in the number of group 3-6 DA clusters in inhibitor-treated embryos. However, the number of cells in the restored DA group never exceeded the number in untreated WT embryos, suggesting that Wnt does not restrict the final population size of group 3-6 clusters (Fig. 6 and see Fig. S5 in the supplementary material). That Dkk1 induced the same proportional increase (two-fold) in DA neurons in the presence or absence of cell cycle blockers, regardless of the developmental stage at which the inhibitors were administered, suggests that Dkk1 does not affect the rate of DA progenitor proliferation.

The observed Dkk1-induced doubling of DA cell number in embryos in which cell division was blocked at 6 hours of development, together with our finding that Wnt inhibition does not delay proper exit of DA progenitors from the cell cycle, suggest that the increase in the number of DA neurons might be due to an effect on early DA progenitors.

Control of DA population size begins during gastrulation

Our fate mapping and analysis of DA progenitor proliferation suggested that Wnt signaling modulates the size of the early DA progenitor pool in the neural plate. To further define the window during which the Wnt/ β -catenin pathway acts to control DA cell number, we used a heat-shock protein 70 (*hsp70*) promoter to induce the expression of Dkk1 at discrete developmental stages. By means of this expression system, we detected ectopic

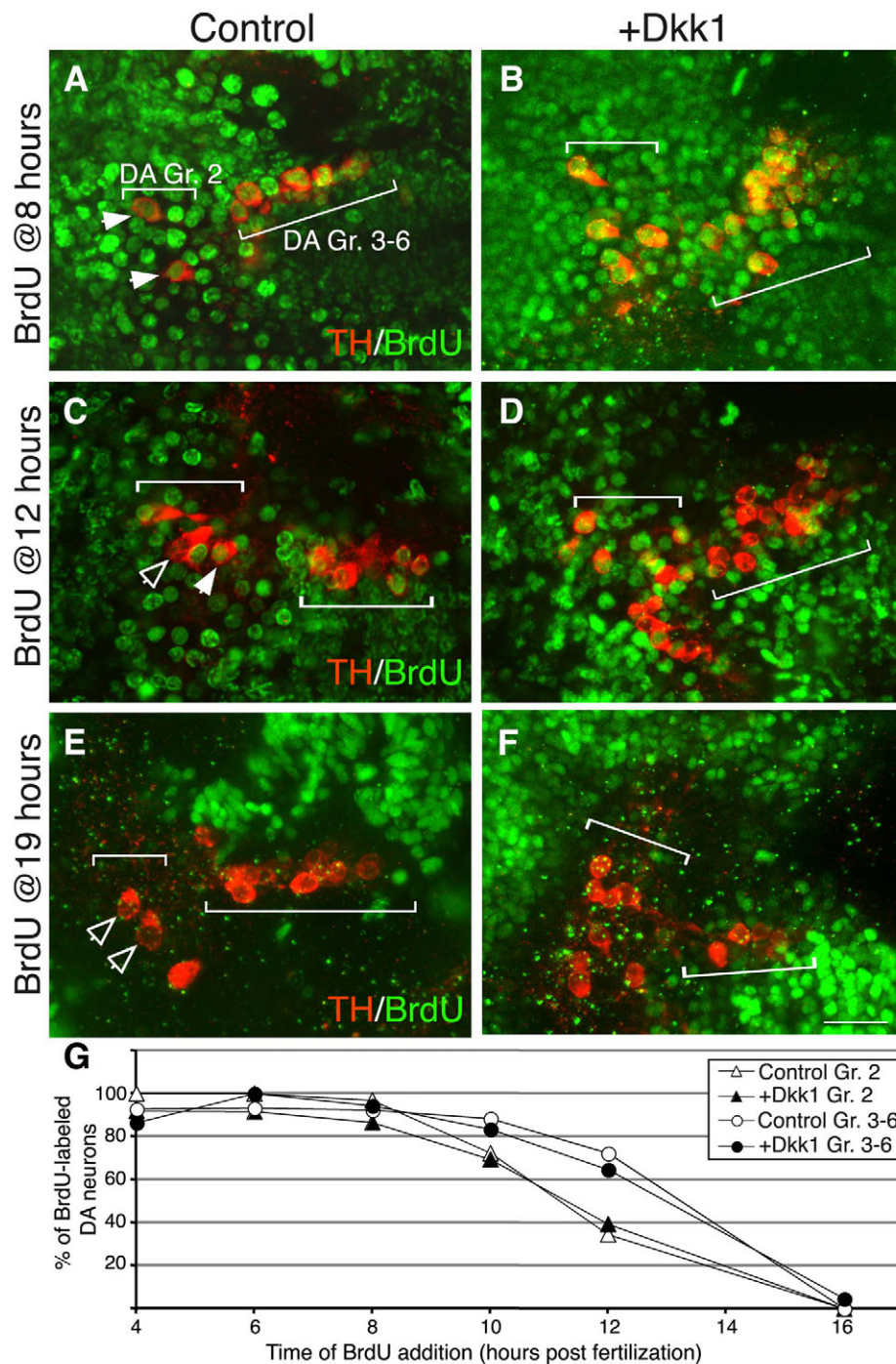


Fig. 5. Dkk1 does not delay cell cycle exit. (A-F) Projected confocal z-stack images of wild-type (WT; A,C,E) and *dkk1*-injected (B,D,F) zebrafish embryos at 48 hpf. Proliferating DA progenitors were labeled by BrdU at the indicated time points followed by immunofluorescence staining with antibodies against tyrosine hydroxylase (TH) and BrdU. The positions of DA group 2 (Gr. 2) and groups 3-6 (Gr. 3-6) are indicated. Solid arrowheads indicate TH⁺ BrdU⁺ double-positive neurons; open arrowheads indicate TH⁺ neurons with no nuclear BrdU staining. (G) Plot of the percentage of proliferating group 2 (Gr. 2; triangles) and group 3-6 (Gr. 3-6; circles) DA progenitor cells as a function of time in WT (white symbols) and *dkk1*-injected (black symbols) populations. Scale bar: 25 μm.

transcription of *dkk1* mRNA as early as 15-30 minutes after shifting the temperature from 28°C to 37°C (data not shown). We also observed that a temperature pulse of 37°C for 30-60 minutes produced the same DA cell number phenotype as that caused by injection of *dkk1* mRNA (Fig. 7A,B). Temporal induction of *dkk1* at various points in development revealed that 6-7 hpf is the latest developmental stage at which Hsp70-driven *dkk1* can elicit a change in DA cell number (Fig. 7C). We conclude that the critical period of competence during which canonical Wnt signaling is able to modulate DA progenitor number occurs between late gastrulation and early somitogenesis, indicating that specification of DA cell number is modulated concomitantly with the establishment of the earliest proneural field.

Wnt8b, Fz8a and Lef1 restrict DA population size

Dkk1 inhibits canonical Wnt signaling by forming a ternary complex with the transmembrane proteins Lrp5/6 and Kremen1/2, thereby reducing the surface levels of these Wnt co-receptors and subsequently blocking signal transduction from the membrane to the nucleus (He et al., 2004). We sought candidate Wnt ligand-receptor complexes that might constitute the target for Dkk1-mediated control of DA cell number. We focused on the Wnt8b ligand and its receptor frizzled 8a (Fz8a; Fzd8a), as both proteins are expressed in the prospective diencephalon both before and during DA differentiation, and are known to be involved in modulating regional fates in the anterior neural plate (Houart et al., 2002; Kelly et al., 1995; Kim et al., 2002). Targeted

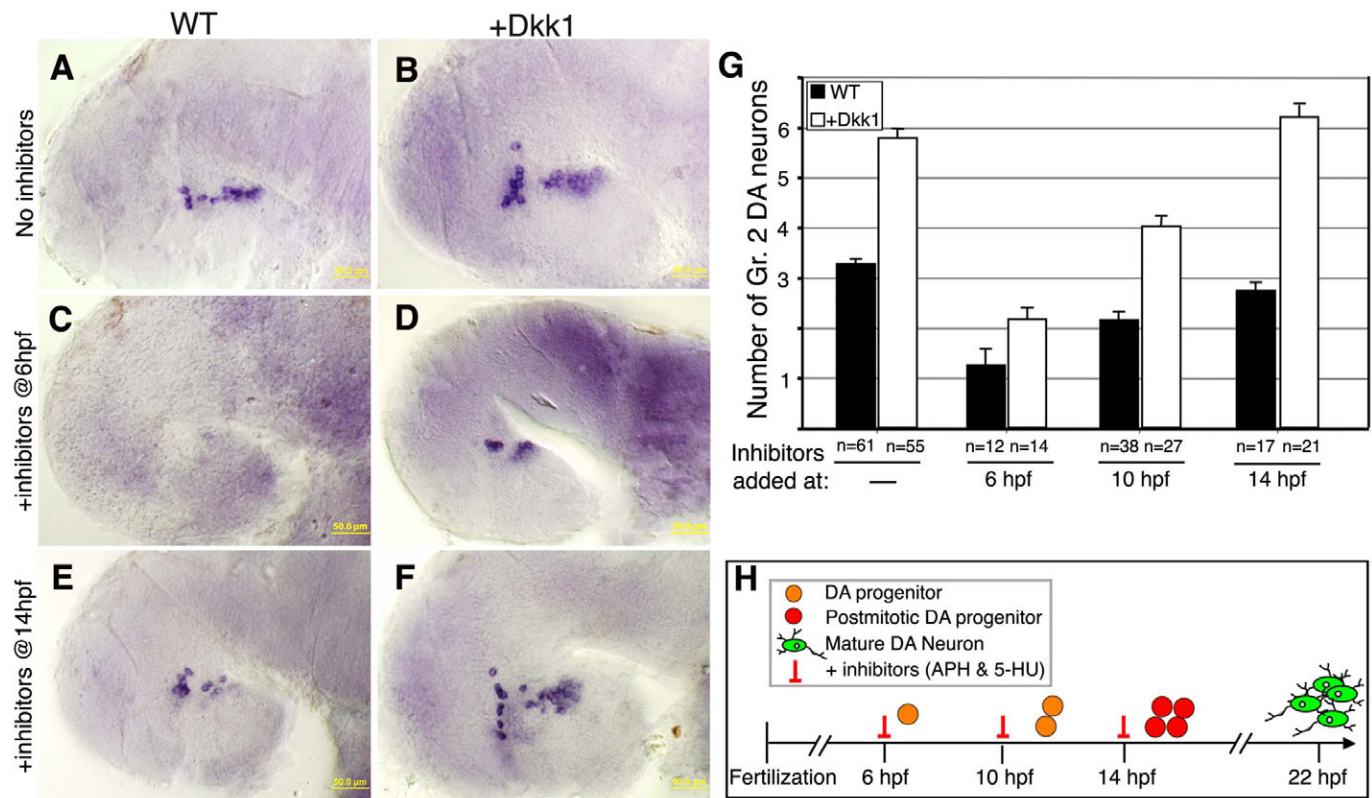


Fig. 6. Temporal inhibition of DA progenitor proliferation. (A–F) Lateral views of *Dat*⁺ DA neurons in zebrafish embryos injected with vehicle (WT; A,C,E) or *dkk1* mRNA (B,D,F). At the indicated time points, embryos were treated with (A,B) solvent (4% DMSO) or with (C–F) a cell cycle inhibitor cocktail containing aphidicolin (APH, 1 μ g/ml) and 5-hydroxyurea (5-HU, 50 mM). DA neurons were counted at 48 hpf. (G) Bar chart showing average cell counts of *Dat*-expressing DA group 2 (Gr. 2) neurons in wild-type and *dkk1*-injected embryos. The number of embryos analyzed (n) is shown beneath. (H) Schematic representation of DA progenitor proliferation, deduced from the analysis of DA cell number following temporal inhibition of cell division. Scale bars: 50 μ m.

knockdown of *wnt8b* using antisense morpholino oligonucleotides (MOs) led to an elevation in DA cell number, phenocopying *dkk1*-injected embryos (Fig. 8B,F). Downregulation of Wnt8b had no effect on the *barhl2*⁺ cell population, which defines a discrete progenitor population juxtaposed to the DA precursor domain (Fig. 8G,H and see Fig. S2 in the supplementary material). MO-based knockdown of *fz8a*, which is known to functionally interact with Wnt8b (Cavodeassi et al., 2005; Kim et al., 2002), led to a similar increase in DA cell number (Fig. 8C,F). Double knockdown of *wnt8b* and *fz8a* did not further enhance the single-morphant phenotype (Fig. 8F), suggesting that the Wnt8b ligand, through the Fz8a receptor, induces a regulatory cascade that limits the number of DA progenitors.

Signaling by Wnt-frizzled ligand-receptor complex initiates a cascade of events leading to activation or repression of Wnt target genes by the β -catenin-Lef/Tcf heterodimer complex (Eastman and Grosschedl, 1999). The Lef1 transcription factor is a crucial determinant of neurogenesis and of neural progenitor fates in the posterior-ventral hypothalamus (Galceran et al., 2000; Lee et al., 2006; van Genderen et al., 1994). Furthermore, both Wnt8b and Lef1 regulate β -catenin-dependent transcription of neural progenitor genes in the basal diencephalon (Lee et al., 2006). To study the possible involvement of Lef1 in the control of diencephalic DA cell number, we quantified DA cells in the *lefl*-deficient mutant, denoted *X8* (Lee et al., 2006), and in the *lefl* morphant. This analysis

revealed that *lefl*-deficient embryos had an elevated number of DA neurons (Fig. 8D–F). Similar to *wnt8b*, inhibition of *lefl* gene activity had no effect on the adjacent *barhl2* expression domain (see Fig. S6 in the supplementary material).

Taken together, these results show that the Wnt8b/Lef1 signaling pathway restricts DA cell number in the PT with no effect on the adjacent *barhl2* expression domain.

fezl (too few) is epistatic to Wnt signaling

We have previously characterized the zebrafish mutant *too few* (*tof*^{m808}), in which there is a marked reduction in cell number of the majority of diencephalic DA clusters owing to a recessive mutation in the gene encoding the Fezl (also known as Fezf2) zinc-finger-containing protein (Levkowitz et al., 2003). Having demonstrated that, like *fezl*, components of the canonical Wnt signaling cascade are necessary in order to maintain the correct number of DA neurons, we next examined whether *fezl* and Wnt(s) share a common genetic pathway.

It was previously shown that the expression of *fezl* in the prospective telencephalon and diencephalon is enhanced following overexpression of Dkk1, indicating that *fezl* expression is repressed by the canonical Wnt/ β -catenin pathway (Hashimoto et al., 2000b). We therefore examined whether *fezl* gene activity is required for Wnt-mediated restriction of DA cell number by inhibiting Wnt signaling in a *fezl*-deficient genetic background. Because *tof*^{m808} is a weak loss-of-function allele (Levkowitz et al., 2003), these mutant

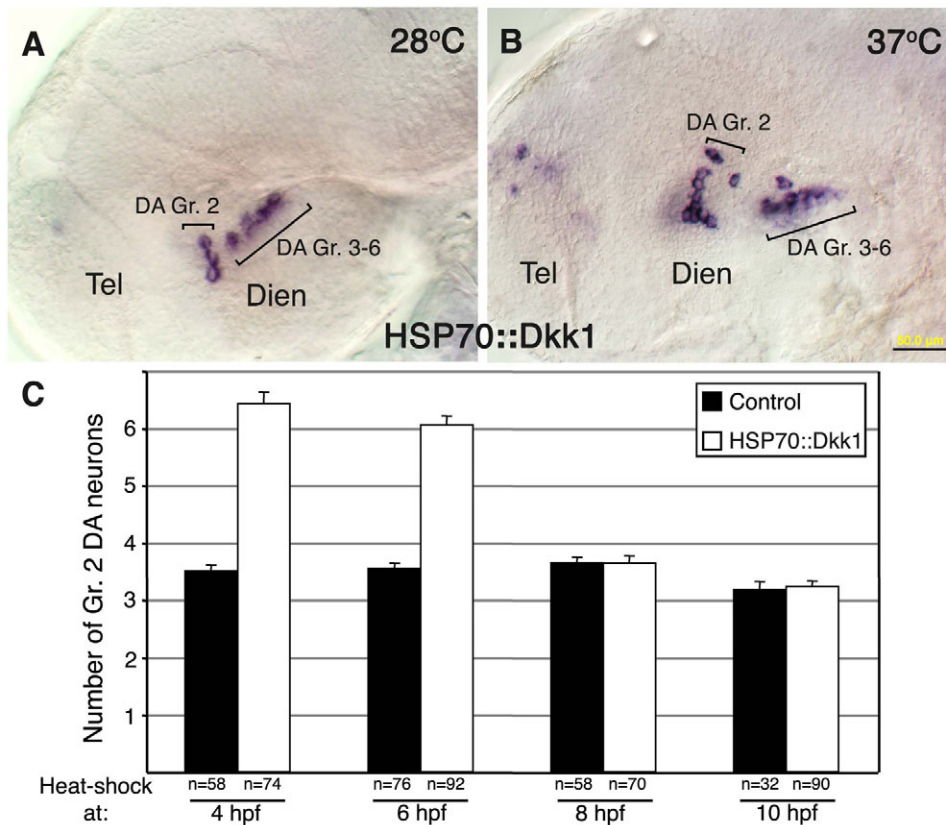


Fig. 7. Wnt attenuation modulates DA cell number at late gastrula stage. (A, B) Micrographs of DA neurons in 48-hpf zebrafish embryos that were injected with a Tol2 vector harboring *dkk1* cDNA under the control of the heat-shock 70 promoter (*hsp70::dkk1*). (A) Embryos were incubated at the non-permissive temperature of 28.5°C for 48 hours. (B) Embryos were incubated at 28.5°C until 4 hpf and were then shifted to 37°C for 1 hour and thereafter grown at 28.5°C. Embryos were subjected (after 48 hours at 28.5°C) to in situ hybridization with a *dat* RNA probe. (C) Bar chart showing average cell counts of DA neurons in control and *hsp70::dkk1*-injected embryos. All embryos were subjected to a 1-hour temperature shock (37°C) at the indicated stages of development, as described above. The number of embryos analyzed (n) is shown beneath. Dien, diencephalon; Tel, telencephalon. Scale bar: 50 μ m.

embryos display a reduction in the group 2 DA cluster; nevertheless, DA neurons are still specified in the *tof^{m808}* mutant (Fig. 9A,D,G,I). We examined whether attenuation of Wnt affected the remaining group 2 DA neurons in *tof^{m808}* mutants. Injection of either *dkk1* mRNA or *wnt8b* MO into *tof^{m808}* embryos did not affect the number of *dat⁺* DA neurons, demonstrating that the effect of Wnt attenuation on DA cells is dependent upon intact *fezl* gene activity and that *Fezl* is a key mediator of DA cell regulation downstream of the Wnt pathway (Fig. 9A-K). Thus, a canonical Wnt signal(s) acts upstream of *fezl*, suggesting that attaining the correct number of DA neurons depends on a balanced activity of Wnt8b and *Fezl*.

Taken together, these results suggest that towards the end of gastrulation, Wnt/ β -catenin activity controls the size of the early diencephalic proneural field, thereby defining the size of the pool of DA progenitors (Fig. 9L). We show that an early canonical Wnt signal, acting through the transcription factor *Fezl*, modulates the number of DA neurons, thereby demonstrating for the first time that early neural patterning cues selectively control the size of the DA neuronal population in the vertebrate diencephalon.

DISCUSSION

In this study, we have explored the effects of localized neural plate patterning signals on the terminal differentiation and configuration of neuronal subtypes in the diencephalon. Surprisingly, perturbation of Wnt signaling did not have an overall effect on specification of diencephalic fates, but selectively affected the number of DA neurons. To further explore this effect, we identified the origin of diencephalic DA progenitors in the neural plate. We then determined the time in development at which these neurons become postmitotic, as well as the developmental stage during which the Wnt/ β -catenin pathway acts to control DA cell number. Our study shows that

Wnt8b/Fz8a-mediated patterning signals act as a restrictive physiological cue controlling DA population size by virtue of their ability to limit the initial pool of diencephalic DA progenitors during early zebrafish development (see proposed model in Fig. 9L).

Relationships between early neural plate patterning, cell identity and cell number

Previous studies have shown that early induction of diencephalic DA fates is governed by patterning cues, such as nodal, *Bmp7*, *Shh* and *Fgf8* (Holzschuh et al., 2003; Mathieu et al., 2002; Ohyama et al., 2005; Ye et al., 1998). The present study demonstrates, for the first time, that the determination of precise numbers of diencephalic DA progenitor cells during primary neurogenesis is specifically controlled by canonical Wnt signaling. Notably, induction of diencephalic DA neuronal identity by *Shh* and *Bmp7* in the chick embryo requires the forebrain-specific transcriptional repressor *Six3* (Ohyama et al., 2005), a protein that in mouse directly binds to the *wnt1* promoter to repress its expression (Lagutin et al., 2003).

One could conceivably attribute the alterations in DA cell number reported herein to the robust action of Wnt on neural patterning. Our study, however, shows that the differential response of distinct neuronal populations to the Wnt signal is not a simple interpretation of their relative anteroposterior position. We propose that diencephalic DA cell number in the PT area is specifically determined by the Wnt8b-Fz8a signaling complex, concomitant with Wnt-mediated regional patterning events. Several lines of evidence support this model. Firstly, we show that attenuation of Wnt signaling leads to a selective expansion of DA cell clusters that are generated in the basal plate of the diencephalon (i.e. the PT; Figs 1-4). Secondly, gain-of-function of several Wnt antagonists at an early developmental stage does

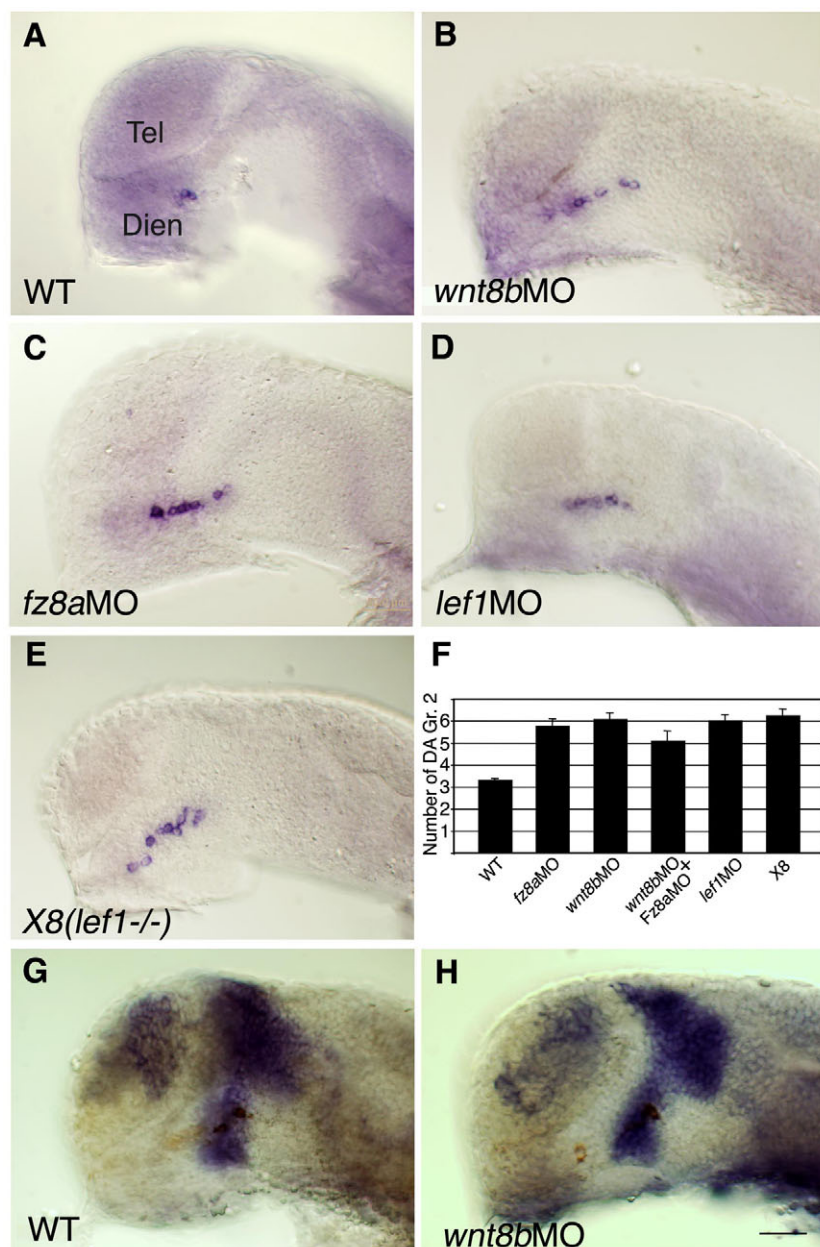


Fig. 8. Canonical Wnt pathway components restrict DA cell number. (A-E) Lateral views of zebrafish embryos that were injected with vehicle solution (WT, $n=71$; A), *wnt8b* morpholino (MO) ($n=20$; B), *fz8a* MO ($n=11$; C) or *lef1* MO ($n=18$; D) and of the *lef1*-deficient X8 deletion mutant ($n=18$; E). Embryos were fixed at 24 hpf and subjected to in situ hybridization with a *dat* probe. The result of the double knockdown of *wnt8b* and *fz8a* ($n=44$) is presented in F. (F) Bar chart summarizing average cell counts of DA group 2 neurons in the genetic backgrounds shown in A-E. (G,H) Lateral views (anterior to the left) of embryos that were injected with vehicle solution (G) or *wnt8b* MO (H). Embryos were fixed at 24 hpf and subjected to whole-mount in situ hybridization with an RNA probe directed against *barhl2*. Scale bar: 50 μm .

not alter the population size or fate of neighboring diencephalic cell types such as IT, SS and Hcrt producing cells (Fig. 2 and data not shown), and of broad diencephalic domains expressing *dlx6a* or *neurog1* (Figs 1, 4). This specificity might be due to differing responses of the neuronal progenitors to the signal, or to a difference in the time of induction of the progenitor pools, which might also determine the nature of the inductive signals to which the progenitors are exposed. Thirdly, Wnt8b/Lef1 activity restricts DA cell number in the PT (Fig. 8), and these embryos have reduced neurogenesis in the adjacent *sox3*⁺ ventral hypothalamic domain (Lee et al., 2006) and normal *barhl2* expression in the neighboring ventral hypothalamus (Fig. 8 and see Fig. S6 in the supplementary material). Finally, inhibiting canonical Wnt signaling in a *tof*^{m808} genetic background led to an enlarged telencephalon without affecting DA cell number (Fig. 9 and data not shown). Hence, the *tof*^{m808} hypomorph enables us to uncouple

the role of Wnt in anteroposterior axis patterning from its role in the control of DA cell number. In a manner similar to Wnt, Fezl might play a role in both neural specification and patterning events (see below).

Cellular mechanisms controlling neuronal cell number: the case of forebrain DA cells

Although it has been suggested that genetic factors play a role in determining DA cluster size (Baker et al., 1980; Baker et al., 1983; Vadasz et al., 1982), the exact mechanisms and cellular events modulating the number of mesencephalic or diencephalic DA progenitors are currently unknown. Zebrafish DA neurons develop extremely rapidly relative to those of other vertebrate species; most diencephalic DA progenitors are in a postmitotic state by 12-14 hpf, and by 4-5 days all DA clusters that appear in the adult brain are already detectable (Fig. 5). These characteristics of the zebrafish

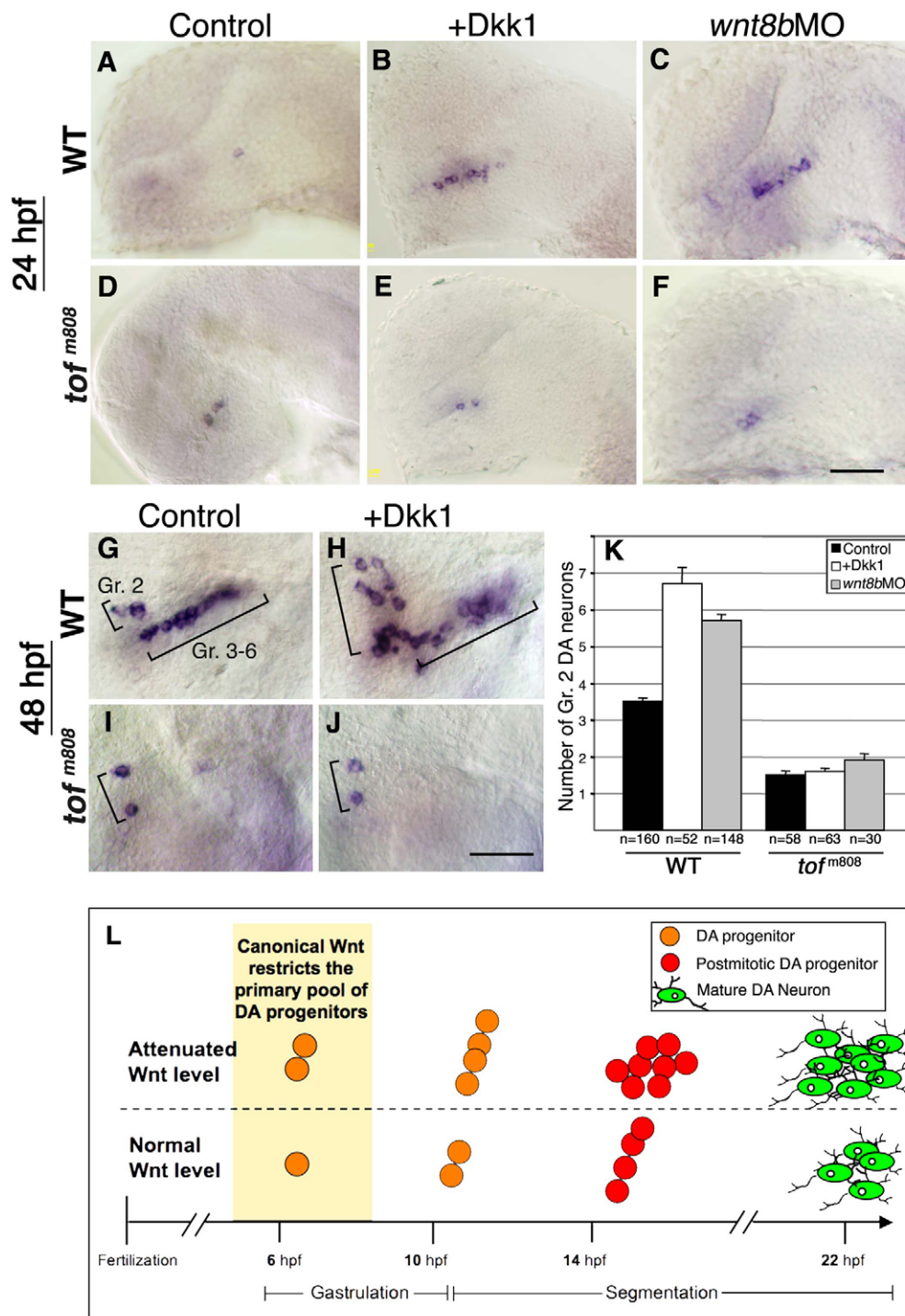


Fig. 9. *fezl* acts downstream of Wnt in controlling DA cell number. (A-F) Lateral views of wild-type zebrafish embryos (WT; A-C) and *too few* mutant embryos (*tofm808*; D-F), which harbor a hypomorphic allele of the *fezl* gene (anterior to the left). Embryos were injected with vehicle solution (control; A,D), *dkk1* mRNA (B,E) or antisense *wnt8b* MO (*wnt8bMO*; C,F). Embryos were fixed at 24 hpf and subjected to in situ hybridization with a *dat* probe. (G-J) Lateral views of wild-type (G,H) and *tofm808* (I,J) embryos. Embryos were injected with vehicle solution (G,I) or *dkk1* mRNA (H,J). Embryos were fixed at 48 hpf and subjected to whole-mount in situ hybridization with a *dat* probe. The positions of DA group 2 (Gr. 2) and groups 3-6 (Gr. 3-6) are indicated. (K) Bar chart presenting average counts of *Dat*⁺ DA group 2 (Gr. 2) cells in WT and *tofm808* embryos that were injected with vehicle solution, *dkk1* mRNA or *wnt8b* MO. The number of embryos analyzed (n) is shown beneath. (L) Proposed mechanism for the control of DA cell number by canonical Wnt signaling. Under normal conditions, early canonical Wnt signals restrict the primary pool of progenitors to a single cell that undergoes two cell divisions before differentiating into DA group 2 neurons. Early attenuation of the Wnt signaling pathway relieves this restriction, allowing two progenitors to divide twice and to differentiate into a significantly larger population of DA neurons. Scale bars: 20 μ m.

brain are compatible with the findings of our study, which is the first demonstration that DA cell number is already tightly regulated at the initial stages of anterior neural plate specification.

The principal mechanisms underlying the regulation of neuronal population size include the control of progenitor proliferation, the regulation of neuronal survival by neurotrophic factors and the self-renewal of neuronal stem cells (Glebova and Ginty, 2005; Johnson, 2003). For the most part, these processes occur during embryonic neurogenesis or in the neonatal brain, after the neural plate has subdivided into prospective rostrocaudal and dorsoventral brain territories. Regulation of neuronal

population size has been mainly studied in the mammalian retina and in the cortex, where it is largely determined by the proportion of neuronal precursors that re-enter the cell cycle (Ohnuma and Harris, 2003; Rakic, 1995). Activation of Wnt/ β -catenin signaling increases the size of the cortex through the propagation of cortical neurogenesis (Chenn and Walsh, 2002; Zechner et al., 2003). However, the increased zebrafish DA population shown herein is not due to a delay in cell cycle exit, an increased proportion of dividing DA progenitors, or a higher rate of proliferation (Figs 5, 6). Analysis of DA cell number following temporal inhibition of Wnt signaling and cell proliferation indicates that the initial pool

of DA progenitors is restricted by the Wnt pathway. These findings suggest that the early embryonic patterning machinery (i.e. Wnt/ β -catenin) also controls the number of neural progenitors that will later assume a DA fate. Thus, the number of DA progenitors is predetermined during primary neurogenesis by patterning signals that control the size of specific neurogenic fields.

The role of the zinc-finger protein Fezl in forebrain and DA neuron development

The forebrain-specific embryonic zinc-finger transcription factor Fezl plays an important role in neural patterning and specification (Chen, B. et al., 2005; Chen, J. G. et al., 2005; Hirata et al., 2006; Hirata et al., 2004; Jeong et al., 2007; Molyneaux et al., 2005). Fezl is one of the earliest markers delineating the prospective forebrain (Hashimoto et al., 2000b; Yang et al., 2001). A hypomorphic allele of *fezl* (*tof*^{m808}) results in a marked decrease in some DA clusters, whereas other diencephalic DA subtypes remain unaffected (Blechman et al., 2007; Levkowitz et al., 2003; Rink and Guo, 2004). Moreover, Wnt8b negatively regulates the expression of Fezl in the neural plate (Hashimoto et al., 2000b). We now extend these findings by showing that Wnt8b acts upstream of Fezl in controlling DA cell number (Fig. 9). The question remains as to whether the canonical Wnt signal regulates Fezl directly or indirectly? Indirect inhibition would suggest that an unknown Wnt/ β -catenin target gene represses *fezl* transcription. A direct repression of *fezl* by Wnt would imply atypical transcriptional repression activity of the β -catenin-Lef1 complex, as was recently shown in the case of pituitary organogenesis (Olson et al., 2006).

Despite evidence for extensive neurogenesis in the adult zebrafish forebrain, the missing DA neurons in *tof*^{m808} mutants are not regenerated in adult animals (Adolf et al., 2006; Grandel et al., 2006; Rink and Guo, 2004). Thus, the activity of Fezl during early neural specification (gastrulation) is crucial in regulating DA cell number. The period of time during which activation of Dkk1 affects DA cell number correlates with the developmental stage at which Fezl affects diencephalic patterning (Fig. 7) (Jeong et al., 2007). Furthermore, it has been shown that the Fezl protein acts upstream of Neurog1, which is the first proneural gene product known to appear in the zebrafish forebrain; moreover, Neurog1 is necessary for diencephalic DA neuron development (Jeong et al., 2006). Similar to Wnt inhibitors, the *tof*^{m808} mutant specifically affects TH⁺ Neurog1⁺ DA neurons in the PT (Blechman et al., 2007). Hence, the genetic interaction between Wnt and Fezl provides a link between early patterning of the forebrain and specific regulation of DA cell number.

In conclusion, our analyses of the role of Wnt activity in diencephalic neuronal subtype decisions reveal a new function for early canonical Wnt signaling in DA neuron development. We present an example of how early neural patterning signals affect the size of a defined neuronal population, concomitantly with the initial regionalization of the neural plate.

We thank Marc Ekker and Uwe Strähle for kindly providing the *dlx6a* and *neurogenin 1* reporter lines; Douglas Lutz for advice and assistance with the two-photon uncaging; Yaara Lancet and Sussane Köthe for technical assistance; members of the Levkowitz laboratory for stimulating discussions; and Avri Ben-Zeev, Mike Fainzilber, Howard Sirotkin, Avraham Yaron and Talila Volk for comments on this work. This study was supported by the Israel Science Foundation (grant number 944/04), National Institute for Psychobiology in Israel, National Parkinson Foundation, Benozio Center for Neurological Disorders, the Dekker Foundation and the Helen and Martin Kimmel Stem Cell Research Institute. G.L. is an incumbent of the Tauro Career Development Chair in Biomedical Research.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/20/3401/DC1>

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