Intracellular trafficking of Notch orchestrates temporal dynamics of Notch activity in the fly brain

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While Delta non-autonomously activates Notch in neighboring cells, it autonomously inactivates Notch through cis-inhibition, the molecular mechanism and biological roles of which remain elusive. The wave of differentiation in the Drosophila brain, the ‘proneural wave’, is an excellent model for studying Notch signaling in vivo. Here, we show that strong nonlinearity in cis-inhibition reproduces the second peak of Notch activity behind the proneural wave in silico. Based on this, we demonstrate that Delta expression induces a quick degradation of Notch in late endosomes and the formation of the twin peaks of Notch activity in vivo. Indeed, the amount of Notch is upregulated and the twin peaks are fused forming a single peak when the function of Delta or late endosomes is compromised. Additionally, we show that the second Notch peak behind the wavefront controls neurogenesis. Thus, intracellular trafficking of Notch orchestrates the temporal dynamics of Notch activity and the temporal patterning of neurogenesis.
N

otch (N) signaling plays diverse roles in many biological processes. N-mediated lateral inhibition is reiteratively used to select a small number of differentiated cells from a large number of undifferentiated cells in a spatially and temporally regulated manner. We previously demonstrated that N-mediated lateral inhibition regulates the speed of proneural wave progression when combined with epidermal growth factor (EGF)-mediated reaction diffusion.

A membrane-bound ligand, Delta (Dl), plays major roles in N-mediated lateral inhibition. It non-autonomously activates N in adjacent cells through a process "trans-activation." Upon binding with Dl, the intracellular domain of N is cleaved to produce the N intracellular domain (NICD), which forms a complex with a DNA-binding transcription regulator, Suppressor of Hairless (Su (H)), and regulates target gene transcription.

On the other hand, N is autonomously inactivated by Dl expressed in the same cell through a process "cis-inhibition," whose molecular mechanism and biological significance remain largely elusive. The direct interaction between Dl and N seems to trigger cis-inhibition by inhibiting N prior to or following its transport to the plasma membrane. There are two possible mechanisms of cis-inhibition. First, the cis-interaction of the ligand and receptor may shut off the transport of N from the endoplasmic reticulum (ER) to the plasma membrane. Second, the cis-interaction may trigger the catalytic process that results in N degradation. For example, the DI–N complex may be internalized from the plasma membrane to cause N degradation. Protein degradation in late endosomes has been shown to play important roles in activating and inactivating N signaling during trans-activation. However, the potential roles of intracellular trafficking of DI and N in cis-inhibition remain largely unknown.

On the surface of the developing fly brain, the wave of differentiation, "proneural wave," propagates along the two-dimensional sheet of neuroepithelial cells (NEs), which sequentially differentiate into neuroblasts (NBs), the neural stem-like cells. In the previous study, we formulated a mathematical model of the proneural wave, which includes N activity (N), DI expression (D), EGF signal activity (E), and the state of NB differentiation (A). A is related to the expression levels of Achaete-Scute Complex proteins (ASC-C). The model successfully reproduses the complex behaviors of the proneural wave in various genetic backgrounds. N is activated along the wavefront, forming an activity peak that negatively regulates the wave propagation. However, N is activated again behind the proneural wave, showing twin peaks of N activity in vivo. If the location of DI-expressing cells does not change, the combination of trans-activation and cis-inhibition would robustly form the twin activity peaks of Notch. However, DI expression propagates as the proneural wave progresses. The mechanism that forms the twin peaks of N activity and the biological significance of the second N activity peak have not been addressed thus far.

Behind the proneural wave, NBs start producing diverse types of neurons. The production of neural diversity is controlled by the transition of temporal transcription factors sequentially expressed in NBs. Homothorax (Hth), Klumpfuss (Klu), Eyeless (Ey), Sloppy paired (Slp), Dichaete (D), and Tailless (Tll) are known to act as the temporal transcription factors in the developing medulla. While Hth expression is already upregulated in NEs prior to NB differentiation, the expression of the other temporal factors is upregulated behind the proneural wave. Thus, the proneural wave could be the initial trigger of the temporal transcription factor cascade following Klu. Since the second N peak is found in NBs behind the wavefront, N signaling could trigger the transition of temporal transcription factor expression.

In this study, we reproduce the twin peaks of N activity by modifying the previous mathematical model and demonstrate that a strong nonlinearity in cis-inhibition robustly reproduces the twin peaks. As a potential candidate mechanism of the nonlinear behavior of cis-inhibition, we assume that DI may transport N to late endosomes, in which Rab7 and the ESCRT (endosomal sorting complexes required for transport) complex quickly degrade N, which results in the inactivation of N between the twin peaks. Indeed, partial knockdown of DI or inactivation of ESCRT complex causes upregulation of N activity and fusion of the twin peaks, forming a single peak of N activity. These results support the idea that intracellular trafficking of N triggers cis-inhibition, which changes the dynamics of N activity. We further explore the biological significance of the twin peaks of N activity. Interestingly, the second N activity peak coincides with Klu expression in the medulla NBs. We demonstrate that Klu expression depends on DI expression along the proneural wavefront, and the formation of the single N peak by inhibiting ESCRT function results in the abnormal temporal patterning of NBs and neurons. Thus, we demonstrate the molecular mechanism of cis-inhibition that orchestrates the temporal dynamics of N activity and the temporal patterning of neurogenesis.

Results

Nonlinear cis-inhibition establishes the twin peaks. In our previous mathematical model, the cis-inhibition term was proportional to DI expression. When the magnitude of trans-activation and cis-inhibition is roughly equivalent, N is activated only once around the wavefront. However, N is activated again behind the wavefront, showing the twin activity peaks (Fig. 1a–d)3,29. We used two independent N activity markers. E(spl)m7GFP (myGFP) shows nuclear signals that are more prominent in the first peak than in the second peak (Fig. 1d)32. In contrast, NREdVenus shows cytoplasmic signals that are more prominent in the second peak than in the other (Fig. 1c)33. If DI is continuously expressed in the same cells, the combination of trans-activation and cis-inhibition would robustly form the twin peaks of Notch activity. However, DI-expressing cells change as the proneural wave progresses. To generate a robust gap between the twin peaks, N must be quickly inactivated just after the first activity peak. Since DI is specifically expressed at the wavefront (Fig. 1b), DI-mediated cis-inhibition could be the cause of the inactivation following the first peak and the formation of the twin peaks of N activity.

We asked if the twin peaks could be reproduced by modifying cis-inhibition in the mathematical model of the proneural wave. It was demonstrated that the kinetics of cis-inhibition are very fast compared with the gradual effect of trans-activation from a series of in vitro experiments. Thus, we incorporated a step function term for cis-inhibition, with which cis-inhibition does not occur when DI expression is below the threshold (c0), but quickly inactivates N when DI expression exceeds the threshold (Fig. 1f). The twin peaks pattern of N activity was reproduced without significantly compromising the magnitude of N activity for a wide variety of parameter settings (Fig. 1e, f and Supplementary Figure 1).

However, the step function is very artificial and nonbiological. According to the previous literature, we incorporated the Hill functions for trans-activation and cis-inhibition. The Hill function is commonly used to model a biochemical reaction, in which the Hill’s coefficient (nH and nH) and activation coefficient (kh and kH) specifies the kinetics of the reaction. We systematically modified the Hill’s and activation coefficients of cis-inhibition to modify its response speed.
and Supplementary Figure 2). The twin peaks pattern of N activity, which is similar to the in vivo pattern (Fig. 1a, c, d), was reproduced when the kinetics of cis-inhibition is faster compared with that of trans-activation. Delta activates Notch activity behind the wavefront. We asked if the mechanism demonstrated in the above in silico experiments exists in vivo. As already demonstrated, the N signal is activated in cells adjacent to a clone of cells ectopically expressing Dl, while it is efficiently inactivated in NE cells expressing DI (Fig. 2a).

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production should cause the fusion of the twin peaks, forming a single N activity peak (Fig. 2b, c). To test this idea in vivo, we made use of two different UAS-Dl RNA interference (RNAi) strains. When expressed in the retina under the control of GMR-Gal4, the mild Dl RNAi reduced the eye width from 0.37 to 0.35 mm, while it was further reduced to 0.31 mm by the strong Dl RNAi (Supplementary Figure 3).

We found that the mild Dl RNAi under the control of optix-Gal4, which is expressed in the dorsal and ventral subdomains of the optic lobe (Fig. 2d), causes the single N peak. The twin N activity peaks were fused to become a single peak (Fig. 2e and Supplementary Figure 4a). When we removed Dl expression by expressing the strong RNAi strain, both of the twin peaks of N activity were eliminated (Fig. 2f and Supplementary Figure 4b) and the same result was also observed in the Dl mutant clone (Supplementary Figure 4c). Note that Dl is specifically expressed at the proneural wavefront cells (Fig. 1b), suggesting that this Dl expression is responsible for the twin activation of N signaling in
N protein is assumed to be constant, while the term \(-k_n N\) represents passive degradation of N signaling, which is independent of DI expression. Considering the above experimental results, full-length N should be actively degraded through DI-dependent \(cis\)-inhibition, which is incorporated into the model as active downregulation of N signaling in a DI-dependent manner (Fig. 1e–g). Our model allows N to be negative (Supplementary Figures 1 and 2), which might correspond to the presence of a repressor complex of Su(H) and Hairless (H)\(^8\). We only use the parameter settings with which N remains nonnegative in the following study.

**Rab7 and Rab4 differentially colocalize with Delta.** It has been reported that DI and N expressed in the same cell form a complex\(^{14,34}\). Consistent observations are found between N and Serrate (Ser), the other transmembrane ligand of N\(^{35}\). The formation of the DI–N complex in \(cis\) may be the cause of the rapid N degradation. It has been reported that protein degradation in late endosomes regulates N signaling\(^{15–24}\). However, these studies did not focus on the process of \(cis\)-inhibition. In order to examine if the degradation machinery in late endosomes is responsible for \(cis\)-inhibition at the proneural wavefront, we asked whether N and DI are transported through the intracellular trafficking pathways.

Rab family small GTPases play diverse roles in intracellular trafficking. We systematically screened the Rab-EYFP library strains in which all of the endogenous Rab family genes were tagged with EYFP\(^{36}\). Since N is downregulated at the wavefront, we initially compared DI expression with Rab-EYFP distribution. We found that Rab4 and Rab7 are colocalized with DI at the wavefront outside the nucleus and inside the membranous Ecad signals (Fig. 3a–f). These puncta are most likely in the cytoplasm. We confirmed the results by using anti-Rab7 antibody and found that DI, Rab4, and Rab7 colocalize in the same puncta (Fig. 3h)\(^{37}\). The proximity ligation assay (PLA) also suggested that Rab7 forms a complex with DI in vivo (Fig. 3g). Since Rab7 and Rab4 are known to play key roles in late and recycling endosomes, respectively\(^{38,39}\), we hypothesized that the DI–N complex is transported to the Rab7-positive late endosomes and that DI is recycled to the plasma membrane through the Rab4-positive recycling endosomes.

Interestingly, we found that Rab4 signals are found inside the DI puncta, while Rab7 mainly accumulates on the surface of the DI puncta at a higher magnification (Fig. 3i). Indeed, the colocalization index of DI-Rab4 was greater compared with that of DI-Rab7 (Fig. 3i, right panel), suggesting that Rab4-dependent DI recycling is more dominant than Rab7-dependent DI degradation. The less prominent colocalization of DI with Rab7 may explain why DI is not degraded in late endosomes.

Although N is downregulated at the wavefront, we occasionally observed minor colocalizations of N with Rab7 and Rab4 (Fig. 3j). The colocalization indices of N and Rab7/4 were significantly lower compared with those of DI and Rab7/4 (Fig. 3k). Importantly, N colocalized with Rab7 more significantly compared with Rab4, suggesting that Rab7-dependent N degradation is more dominant than Rab4-dependent N recycling. Similarly, colocalization of DI-GFP, which recapitulates DI distribution pattern, and N was occasionally observed (Fig. 3l and Supplementary Figure 5c). These results support the hypothesis that N is mainly degraded in late endosomes at the wavefront in a DI-dependent manner.

**ESCRT complex downregulates Notch activity at the wavefront.** When proteins are transported from early to late endosomes (or multivesicular bodies), Rab7 and the ESCRT complex (ESCRT-I–III) regulate protein degradation through the fusion of late endosomes with lysosomes\(^{21,38}\). Since DI colocalizes with
Rab7 at the wavefront, we asked if the DI–N complex is transported to late endosomes and N is degraded therein. Many studies have reported the multiple roles of late endosomes in N signaling. When the function of late endosomes is compromised, the expression of DI and N is upregulated. As a result, N signaling is ectopically activated. On the other hand, it is also known that late endosome function is required for N activation. Indeed, N activity is downregulated...
when the function of the ESCRT protein is compromised in some experimental conditions. Thus, late endosomes appear to be involved in multiple aspects of N signaling.

When we knocked down rab7 by expressing the dominant-negative form of rab7 or rab7 RNAi, N protein level was slightly upregulated, showing punctate signals as visualized by N antibody (Figs. 4a, b, j and 5a, n). N and DI colocalized with the Rab7DN puncta (Fig. 4a–d). However, the N activity reporter was not significantly affected when rab7 was knocked down (see Fig. 5a).

Other late endosomal components may act redundantly with Rab7. We, therefore, focused on the functions of Vps family proteins that are involved in ESCRT complex function. When we knocked down vps2, a member of ESCRT-III, at the wavefront, N protein level was also upregulated, showing punctate signals (Fig. 4e, f). N and DI colocalized in the Rab7- and Rab4-positive puncta (Fig. 4e–i).

We observed two distinct outcomes in N activity in the vps2 RNAi background. In 40% of the cases (n = 8/20), N activity was specifically upregulated in cells situated between the twin peaks nearby the wavefront indicated by Lsc expression. The level of full-length N protein was slightly upregulated forming a dotted pattern. At the same time, the twin peaks were fused to form a single peak (Figs. 4e, g and 5d–f, n and Supplementary Figure 6a), which is similar to the results of the partial knockdown of DI shown above (Fig. 2e and Supplementary Figure 4a). Since the upregulation of the level of full-length N were not as prominent as those found in DI mutant clones (Fig. 2i, j), there might be as yet unknown mechanisms that regulate N degradation and cis-inhibition. Importantly, DI expression level was not significantly affected (Fig. 4g, j and Supplementary Figure 6b), suggesting that N activation and the formation of the single peak are not caused by changes in DI expression.

Since EGF signaling indirectly influences N activity, the above phenotype may be the result of the changes in EGF activity. Since EGF activity as visualized by PntP1 staining was not significantly affected, the N activation phenotypes discussed above were not caused by indirect effects through EGF signaling (Supplementary Figure 7).

In the other 60% of the cases, DI and full-length N proteins were widely upregulated (Figs. 4h, j and 5m and Supplementary Figure 6c, d, n = 12/20). Low-level N activity was observed in a wide area encompassing the wavefront (Fig. 5m and Supplementary Figure 6c), which may be related to the hyper-activation of N.
signaling and/or suppression of N activity in vps2 mutant cells. Since these phenotypes accompany smaller brain sizes compared with the brains showing the specific N activation phenotype discussed above, we assume that the mild RNAi effect caused the specific N activation between the twin peaks (Fig. 5d).

We repeated the same experiments for the other ESCRT complex genes and found essentially the same results (Fig. 5g–n and Supplementary Figure 8). Importantly, the specific N activation at the wavefront and the fusion of the twin peaks were reproducible in multiple RNAi conditions and mutant conditions.
clones of vps2 and vps22 (Fig. 5g–p and Supplementary Figure 8). Note that the twin peaks were partially fused, while ectopic N activation was not found in cells apart from the wavefront in the vps2 and vps22 mutant clones (Fig. 5o, p; n = 9/15 and 11/16). These results cannot be explained by the previously proposed roles of late endosomes that nonspecifically and uniformly degrade N and Dl. Thus, late endosomes play specific roles in N degradation that are essential for cis-inhibition at the proneural wavefront in addition to the roles in uniform nonspecific N degradation.

Delta transport from late endosomes to recycling endosomes.

We hypothesize that the Dl–N complex is transported to late endosomes, where only N is degraded (Fig. 6o). We also hypothesize that Dl is released from late endosomes prior to its degradation and is recycled to the plasma membrane through recycling endosomes, because Rab4 colocalized with Dl more significantly compared with Rab7 (Fig. 3i).

When we knocked down rab4 with RNAi, Dl expression was accumulated at the wavefront in a milder condition at 25 °C (Fig. 6a, b, m) and the colocalization of Dl with Rab7 was significantly increased (Fig. 6b). Interestingly, Dl expression was downregulated in a stronger RNAi condition at 30 °C (Fig. 6c, m). These results suggest that Dl is retained in Rab7-positive late endosomes and is degraded together with N when the function of recycling endosomes is eliminated. Furthermore, overexpression of rab4DN mimicked the effects of rab4 RNAi in the milder condition, and aggregated distribution of Dl colocalized with Rab7 in the cytoplasm of cells at the wavefront (Supplementary Figure 9). Similar results were demonstrated in cells mutant for the components of recycling endosomes in the central brain41.

It is known that late endosomes become acidic in the course of fusion with lysosomes, by which proteins are degraded42. The decrease in pH may trigger Dl dissociation from the DI–N complex. Rabconnectin (Rbcn) is a family of proteins that regulate endocytic trafficking by regulating the assembly and activity of vacuolar-ATPase (V-ATPase), which is responsible for the acidification of intracellular compartments43,44. Thus, we focused on the roles of Rbcn and vha68-2, a member of V-ATPase genes, in this process. In vha68-2 mutant clones, DI was aggregated and colocalized with Rab7 (Fig. 6d, e). When vha68-2 was knocked down, signals indicating acidic cellular components were significantly reduced as visualized by Lysotracker (Supplementary Figure 10c). At the same time, N degradation was blocked, and the level of N protein was upregulated at the wavefront (Fig. 6f–j and Supplementary Figure 10). In this condition, we observed ectopic colocalization of DI and N in Rab7 and Rab4-positive puncta (Fig. 6h–i), suggesting that acidification of the endocytic pathway triggers DI dissociation from the DI–N complex and N degradation. These results are consistent with the impaired endosomal acidification and accumulation of N in late endosomes and lysosomes in vha68-2 mutant clones in eye imaginal disc45.

Based on the above results, we hypothesize that the DI–N complex in Rab7-positive late endosomes is dissociated upon pH decrease. As a result, N is degraded in lysosomes, while DI is recycled to the cell membrane through Rab4-positive recycling endosomes. Since pH increase upon vha68-2 RNAi caused DI colocalization with Rab7 and Rab4 (Fig. 6h, i), DI–N complex may be localized to either late endosomes or recycling endosomes when acidification is compromised.

If DI is transported from late to recycling endosomes, DI should more strongly colocalize with Rab7 when rab4 is knocked down together with vha68-2. To test this possibility, we compared colocalization of DI with Rab7 in vha68-2 RNAi and vha68-2 rab4 double RNAi backgrounds. Indeed, the colocalization of DI with Rab7 was significantly increased by knocking down rab4 together with vha68-2 (Fig. 6h, k, l, n). These results support the model shown in Fig. 6o.

The second Notch activity controls neurogenesis.

The first peak of N activity is responsible for regulating the speed of proneural wave propagation3,27. What is the biological function of the second peak? We carefully compared the pattern of N activity and genes that are specifically expressed in the NBs behind the wavefront. Interestingly, the expression of one of the temporal transcription factors, Klu, coincides with the second peak of N activity (Fig. 7a, l)31,46.

So far, Hth, Klu, Ey, Slp, D, and Tll show sequential expression in the medulla NBs30,31. However, the mutual interactions between Hth, Klu, and Ey have not been identified thus far. Since Hth is upregulated in NEs prior to the arrival of the proneural wave and is continuously expressed in NEs and NBs (Fig. 7l), the mechanism that regulates Klu expression remains unclear. The N activity in the second peak may regulate the onset of Klu expression in NBs.

We initially examined the effect of the complete elimination of DI function by generating DI-null mutant clones in which the proneural wave is accelerated27. Since Klu expression persists for a long time following its induction at the second N activity peak, the premature NB differentiation would still show a persistent Klu expression behind the accelerated wavefront. However, Klu expression was eliminated in DI mutant cells except for the cells situated along the boundary between the DI-positive cells (Fig. 7b, n = 16). The residual Klu expression along the clone boundary may be due to the non-autonomous effect of N trans-activation. In contrast, the expression Hth, Ey, and Slp was not significantly affected in DI mutant clones (Supplementary Figure 11a–c). These results suggest that N signaling is indeed necessary for Klu expression in NBs.

Since we do not have a technique that specifically inactivates the second N peak, we made use of the partial knockdown of DI and vps2, resulting in the loss of the gap between the twin peaks and, consequently, the fusion of the twin peaks. Interestingly, Klu was precociously expressed in the newborn NBs under these conditions without significantly affecting the proneural wave progression (Fig. 7c, d), suggesting that the second N activity peak in NBs indeed triggers the expression of Klu. Note that Klu expression is not activated in the first peak of N activity in NEs (Fig. 7a, l). Klu expression may require additional genetic factors that are specific to NBs.

We previously demonstrated that Hth expression in the newborn NBs promotes the production of brain-specific homeobox (Bsh)-positive neurons that form the innermost concentric zone in the larval medulla (Fig. 7c, f, l)47,48. In slightly older NBs, Klu expression triggers the production of Runt (Run)-positive neurons, forming a concentric zone just outside the Bsh-positive neurons31. Consistent with the precocious Klu expression in NBs, we occasionally observed Run-positive neurons in an area inside the Bsh-positive neurons in DI and vps2 RNAi conditions (Fig. 7g, h, i). These defects were restricted to the dorsal part of the brain, most likely due to the stronger expression of optix-Gal4 in the dorsal brain (Fig. 7e). The Klu expression in the newborn NBs, which only express Hth in the control background, might cause the production of Run-positive neurons earlier than Bsh-positive neurons, resulting in their abnormal distributions in the medulla (Fig. 7l). Note that Hth expression is widely found in NE and NB cells, and is not affected by Klu31. Therefore, the expression of Hth and production of Bsh-positive neurons should not be affected.
We have demonstrated that Bsh-positive neurons give rise to Mi1 medulla neurons.7,48 Similarly, the results of clonal analysis using run-Gal4 demonstrate that Run-positive neurons differentiate into Mi4 and TmY16 neurons (Fig. 7j, k; n = 60 and 49, respectively). The neuronal-type TmY16 has not been documented based on its projection pattern in the medulla, lobula, and lobula plate (Dr. Kazunori Shinomiya, personal communication). Thus, the temporal regulation of the N dynamics at the proneural wavefront controls the temporal pattern of neuronal-type specification through the expression of the temporal transcription factor Klu.

**Discussion**

In this study, we incorporated nonlinearity in cis-inhibition to the mathematical model of the proneural wave and reproduced the twin activation peaks of N signaling at the wavefront as observed in vivo. The fast nonlinear dynamics of cis-inhibition compared...
with those of the gradual kinetics of trans-activation is consistent with the results of the in vitro cell culture system\(^1\). The differential kinetics of trans-activation and cis-inhibition provide the rich dynamics of N activity that enable the formation of the twin activation peaks and the regulation of the temporal patterning of neurogenesis.

According to the previous literatures, the upregulation of Dl expression may induce the clustering of N and Dl\(^1^4,3^4,3^5\), which leads to an acute suppression of N signal activity via cis-inhibition (Fig. 6o). Consistent with this idea, the partial knockdown of Dl caused an upregulation of N activity between the twin peaks and their fusion, which reflects the failure in cis-inhibition. The expression level of full-length N is temporally downregulated behind the wavefront, which is derepressed in Dl mutant clones (Fig. 2h, i), suggesting that cis-inhibition is triggered by N degradation in response to Dl expression.

We essentially focused on the roles of intracellular trafficking in regulating N degradation and cis-inhibition (Fig. 6o). RNAi and mutant clones of ESCRT complex genes caused the fusion of the twin peaks of N activity, which is similar to the result of the partial Dl knockdown. We assume that the ESCRT complex regulates N degradation in the presence of high levels of Dl.
expression. Since the expression level of full-length N was only partially upregulated, forming a dotted pattern by knocking down rab7 and ESCRT genes, there might be as yet unknown mechanisms that regulate N degradation (Fig. 5, Supplementary Figure 6).

We assumed that the clustering of N and DI is the trigger of N degradation. However, DI is not degraded at the wavefront, as evident from its strong membrane localization, despite its localization in Rab7-positive late endosomes. Since the colocalization of DI with Rab4 is more prominent than that with Rab7, recycling of DI to the plasma membrane may be dominant compared with its degradation (Fig. 3i). Indeed, a mild knockdown of DI caused DI accumulation in Rab7-positive late endosomes, while its severe knockdown induced DI degradation (Fig. 6a-c). Colocalization of DI with Rab7 upon blocking acidification in late endosomes was further enhanced by the simultaneous knockdown of vha68-2 and rab4 (Fig. 6l, n). These results suggest that DI is dissociated from the DI–N complex in late endosomes, and is recycled to the plasma membrane through recycling endosomes (Fig. 6o).

The mathematical models in Fig. 1 do not explicitly consider the degradation of N protein upon its cis-interaction with DI. We further improved the model by considering full-length N (F) and active form of N (S). In a wide range of parameter settings, this model reproduces the formation of the twin peaks of N activity, fast degradation, and gradual recovery of the expression level of full-length N (Supplementary Figure 12). Although DI function is thought to be inhibited when DI and N interact in cis, we did not include this reaction in the model, because we do not have any observation that suggests cis-inhibition of DI in the course of the proneural wave progression.

Furthermore, we demonstrated a role of N signaling in regulating the temporal patterning of neural progenitor cells (NPCs) by focusing on the transition of the temporal transcription factor Klu, whose expression is upregulated in the medulla NBs. We showed that DI remotely regulates Klu expression behind the proneural wave through the second peak of N activity. Thus, fine temporal regulation of N activity through cis-inhibition plays essential roles in the temporal patterning of neurogenesis in the fly brain.

In the NPCs of the developing cerebral cortex, the temporal dynamics of N activity also play important roles in the temporal patterning of neurogenesis and gliogenesis. In this process, the basic helix–loop–helix transcription factors show oscillatory expression in NPCs and N signaling appears to perform lateral inhibitory feedback during NPC differentiation. The roles of cis-inhibition in this process remain largely elusive. It will be interesting to see how the molecular mechanisms revealed in the current study are conserved in a wide variety of developmental processes.

**Methods**

**Mathematical modeling and numerical simulation.** The differential equations were calculated using the explicit finite difference method with the zero flux boundary condition as described previously. The mesh size of the square grid model is equal to the cell size (dx = 2, 25 × 25 cells), and the time step size is 0.01.

Four-variable model. The model contains four equations and four variables. E is a composite variable for EGF ligand concentration and EGF signaling. N represents N signal activity. D and A represent expression levels of DI and AS-C, respectively. di is the diffusion coefficient of EGF. di and di represent magnitudes of the trans-activation and cis-inhibition, respectively. j, i, and k, m are integers indicating the location of a cell along the x and y axes, respectively. l and m indicate the location of four cells adjacent to a cell indicated by j and i. The initial conditions for A, E, and D in the anterior-most cells (x = 1, 2, 3) are A = 0.90, 0.31, and 0.02; E = 0.054, 0.021, and 0.0016; and D = 0.062, 0.021, and 0.0013, respectively, to stabilize the proneural wave progression. The initial condition of N is 0 in all cells.

The diffusion of E was calculated as follows: dE/dt = dE/dx + dE/dy + dD/dt = ddx(dx) + E + E + E + E, where k, k, and k are passive degradation rates of EGF, N, and DI, respectively. ai and aj indicate EGF and DI regulation by AS-C, respectively. When A = 1, the cells are fully differentiated as NBs. ci reflects the speed of differentiation under the control of EGF and N, and is set to 100. The other parameters are set to 1 unless otherwise noted.

Linear cis-inhibition: In the previous model, the trans-activation and cis-inhibition are linear (1–2). k = 1, 0.25, and 0.05. In this study, (1–2) is replaced with the following.

\[ dA/dt = -kA/Di + aA(k/A - 1) \quad (1-4) \]

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Lambda cis-inhibition: In the previous model, the trans-activation and cis-inhibition are linear (1–2). k = 1, 0.25, and 0.05. In this study, (1–2) is replaced with the following.

\[ dA/dt = -kA/Di + aA(k/A - 1) \quad (1-4) \]

The diffusion of E was calculated as follows: dE/dt = dE/dx + dE/dy + dE/dt = ddx(dx) + E + E + E + E, where k, k, and k are passive degradation rates of EGF, N, and DI, respectively. ai and aj indicate EGF and DI regulation by AS-C, respectively. When A = 1, the cells are fully differentiated as NBs. ci reflects the speed of differentiation under the control of EGF and N, and is set to 100. The other parameters are set to 1 unless otherwise noted.

**Hill function: (1–2) is replaced with the following.**

\[ H(x) = \begin{cases} 1 & (x \geq c_2) \\ 0 & (x < c_2) \end{cases} \quad (2-2) \]

Hill’s coefficient (n) and activation coefficient (k) and k, k, and k, k specify the kinetics of the reaction. k = 1, d = 0.25, d = 0.1, n = 1, k = 1, n = 5, and k = 0.09 (Figs. 1g and 2c). Hill’s coefficient (n) and activation coefficient (k) of cis-inhibition are changed in the
range 2–7 and 0.07–0.11, respectively (Supplementary Figure 2).

\[
\frac{dN_i}{dt} = -\lambda N_i + \frac{d}{k_i} \left( \sum_{m=1}^{n} D_{m,i} \right) + \frac{d}{k_i} \frac{D_{i,i}}{k_i} - \frac{d}{k_i} \frac{D_{i,i}}{k_i}
\]

(3–1)

Five-variable model: (1–2) is replaced with (4–1) and (4–2). F and S represent the expression level of full-length N and N signal activity, respectively, whose initial conditions are 1 and 0 in all cells. \( F_{\text{max}} \) and \( n \) represent maximum expression level, recovery rate, and spontaneous degradation rate of full-length N, respectively. \( d_i \) and \( d_i \) represent magnitudes of the trans-activation and cis-inhibition, respectively. Hill’s coefficient (\( n \)) and activation coefficient (\( K \)) specify the kinetics of the reaction. \( k_i \) represents the degradation rate of full-length N upon trans-activation. \( k_i \) represents passive degradation rate of active form of N. \( n_i = 0, 1, 2, 3, \) and \( k_1 = 0.1, 0.2, 0.3, \) and \( k_2 = 0.25, \) and \( k_3 = 0.25. \) Hill’s coefficient \((n)\) and activation coefficient \((K)\) for Hill function in cis-inhibition are changed in the range 1–7 and 0.05–0.3, respectively (Supplementary Figure 12).

\[
\frac{dF}{dt} = \frac{n \left( F_{\text{max}} - F_i \right) - k_i F_i}{k_i + \left( \sum_{m=1}^{n} D_{m,i} \right)}
\]

(4–1)

\[
\frac{dS}{dt} = \frac{k_F F_i \left( \sum_{m=1}^{n} D_{m,i} \right)}{k_i + \left( \sum_{m=1}^{n} D_{m,i} \right)} - k_i S_i
\]

(4–2)

Two- and one-dimensional plots in Figs. 1–2b, c, and Supplementary Figures 1, 2, and 12 are the snapshots when the state of differentiation of the central cell exceeds 0.5 (A111, > 0.5). The source codes for the numerical simulations will be deposited to a public repository service.

Fly strains. Fly strains were maintained on standard Drosophila medium at 25 °C. rab5 RNAi was performed at 25 and 30 °C. The following mutant and transgenic were used: D3FL0053 FRT82B, UAS-Dp(1) FRT82B, UAS-DNAI (strong: BSC63784; mld: VS25818), Ir-GFP (BSC59819), rab7-EYFP (BSC62542), UAS-rab4-EYFP (BSC7778), UAS-rab7 RNAi (BSC77051), VDRC03383, UAS-rab4DNAI (BSC7968, BSC7969), vps23 FRT82B, vps23RNAI (BSC38995), UAS-vps2 RNAI (BSC38289), UAS-vps23 RNAI (BSC38306), UAS-vps25 RNAI (BSC26286), UAS-vps24 RNAI (BSC38281), UAS-vps32 RNAI (BSC38305), UAS-vps20 RNAI (BSC38949), UAS-vps36 RNAI (BSC38286), UAS-vps35 RNAI (BSC4010), N163-ubtub-2 RNAI (BSC43582), w64a6b-2fRT82B (BSC39621), uas-StatC RNAI (BSC34612), optix-Gal (Np2631), Ay-Gal4, UAS-CD8GFP, UAS-GFP, UAS-hfp, ubi-GFP FRT82B, and ubi-RFP FRT82B. N activity was visualized by Espl1-myGFP (myGFP) and NRE-4hem2.2b.

Clonal analysis. Dp insertion clones were generated by crossing hs-flp; Ay-Gal4 UAS-GFP strain with UAS-Dp(1) FRT82B and applying 15 min heat shock at 34 °C. Dp mutant clones were generated by crossing hs-flp; ubi-GFP FRT82B strain with D3FL0053 FRT82B and applying 50 min heat shock at 37 °C. vps23 mutant clones were generated by crossing hs-flp; myGFP FRT82B and vps23 FRT82B and applying 50 min heat shock at 37 °C. vps20 mutant clones were generated by crossing vps20 RNAI FRT82B, FRT82B, vps20 RNAI FRT82B, and vps20 RNAI FRT82B with vps23 FRT82B, vps23 RNAI FRT82B, vps23 RNAI FRT82B, and vps23 RNAI FRT82B. N163-ubtub RNAI (BSC43582), w64a6b-2fRT82B (BSC39621), uas-StatC RNAI (BSC34612), optix-Gal (Np2631), Ay-Gal4, UAS-CD8GFP, UAS-GFP, UAS-hfp, ubi-GFP FRT82B, and ubi-RFP FRT82B. N activity was visualized by Espl1-myGFP (myGFP) and NRE-4hem2.

Generate run-Gal4 strain. The second exon containing the translational start site of run was replaced by the fragment containing attP site and GMR-white via FLP transposition. To generate the run-Gal4 strain, the fragment containing the translational start site of run was inserted into the empty site of P-element system. The following primary antibodies were used: guinea pig anti-Lsc (1:1200), mouse anti-DI (1:20; DS85 C3949), mouse anti-N (1:20; DS85 C1796), and rabbit anti-Rab7 (1:600; Akira Nakamura, Kumamoto University, Japan). The following secondary antibodies were used: anti-mouse Cy3 (1:20; Jackson ImmunoResearch 715-165-151), anti-mouse IgG (Alexa467 (1:200; Jackson ImmunoResearch 712-605-150), and anti-rabbit Alexa546 (1:200; Invitrogen A-11035). Confocal images were obtained by Zeiss LSM880 and processed using ZEN, Fiji, and Adobe Photoshop. Signal intensity was quantified within the indicated rectangular areas by ImageJ.

In situ hybridization. In situ hybridization was performed as briefly described below. Details are available upon request. Larval brains were dissected in ice-cold PBS, transferred to 4% formaldehyde/PBS solution, and fixed at 4 °C overnight. The formaldehyde solution was removed, and the brains were washed with PBS and 70% ethanol. Incubation at room temperature for 2–5 min after replacing the solution with Wash Buffer A (100 μl) Starlight RNA FISH Wash Buffer A, 300 μl nuclelease-free water, 50 μl deionized formamide. Replacement with the Hybridization Buffer (90 μl Hybridization Buffer mix with 2 μl Starlight RNA FISH probe designed for exon 6 of N gene labeled with Quasar 570), the brains were incubated at 37 °C for 8 h. Incubation at 37 °C for 30 min after replacing the solution with Wash Buffer A. Replacing the solution with TO-PRO3/PBS, the brains were incubated at 37 °C for 30 min. After washing in PBS, the brains were mounted in glycerol.

Duolink PLA. PLA was performed using Duolink (Santa-Cruz). The dissected fly brains were fixed in 4% formaldehyde in PBT (0.3% Triton X in PBS). After finishing the Lsc immunostaining, the brains were incubated with Duolink blocking solution for 60 min at 37 °C and in the Duolink antibody dilute containing mouse anti-DI and rabbit anti-Rab7 antibodies for overnight at 4 °C. The brains were washed in Duolink Wash Buffer A three times at room temperature, incubated in a solution containing PLA PLUS anti-mouse and PLA MINUS anti-rabbit probes for 120 min at 37 °C, washed in Duolink Wash Buffer A, and incubated with Duolink ligation stock for 60 min at 37 °C. Then, the brains were washed in Wash Buffer A and incubated with Duolink polymerase in Duolink amplification stock for 100 min at 37 °C. Finally, the brains were washed in Wash Buffer B and mounted in Duolink in situ mounting medium with DAPI and 4,6-diamidino-2-phenylindole.

LysoTracker staining. Fly brains were dissected in cold S2 medium and incubated in 50 ng/ml of LysoTracker Red DND99 (BDSC 40894), which contains DND99 (BDSC 40894), the brains were incubated in a solution containing secondary antibodies and 1% normal saline (PBS), transferred to ice-cold 0.8% formaldehyde/PBS solution, and fixed in 4% formaldehyde/PBS at room temperature for 30–60 min. The brains were washed in PBT (0.3% Triton X in PBS) and blocked in 5–10% normal serum/PBT solution at room temperature for 30–60 min. Primary antibody reaction was performed in a solution containing primary antibodies and 1% normal serum in PBT at 4 °C overnight. The brains were washed in PBT. Secondary antibody reaction was performed in a solution containing secondary antibodies and 1% normal serum in PBT at 4 °C overnight. After washing in PBT and PBS, the brains were mounted in VECTASHIELD (Vector Laboratories).

Image quantification. Signal intensity was quantified within the indicated rectangular areas using Fiji (Figs. 1–c, d–e, i, 4, 5, and 6f–n). Coloc 2 function of Fiji was used to calculate the Pearson’s R value to quantify the colocalization between two different signals (Figs. 3b, k, and 6f, n). The coordinates of cells were obtained by manually selecting individual expressing Run or Bsh (Fig. 7g, h). The coordinate of the origin was calculated as the average of cell locations along the x-axis. The y coordinate of the origin was determined so that the standard deviation of R, the distance from the origin to cells, is minimized. \( \theta \), the angle of mutant area from the origin, was determined based on the mutant area of Bsh-positive cells. The same \( \Delta \) was used to determine the control area to be analyzed at the dorsal–ventral boundary where optix-Gal is not expressed. Sizes of the intersection between Run- and Bsh-positive areas in the control and mutant areas were calculated using “convhull,” “polyshape,” and “intersect” functions of MATLAB (Fig. 7i).

Ethical approval. We have complied with all relevant ethical regulations for animal testing and research. This study did not require an ethical approval.

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-22442-3 | www.nature.com/naturecommunications

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The authors declare that the data supporting the findings of this study are available within the paper, or available upon request. Source data are provided with this paper.
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**Acknowledgements**

We thank members of Sato lab for supporting fly work, T. Kawauchi, K. Matuno for helpful discussion, S. Bray, A. Nakamura, J. Skeath, Bloomington Drosophila Stock Center, Vienna Drosophila Resource Center, Drosophila Genetic Resource Center, Kyoto, Asian Distribution Center for Segmentation Antibodies, Mishima, and Developmental Studies Hybridoma Bank for flies and antibodies. This work was supported by CREST from JST (JPMJCR14D3 to M.S., S.-I.E. and JPMJCR15D2 to M.N.), Grant-in-Aid for Scientific Research (B), (C), Grant-in-Aid for Scientific Research on Innovative Areas and Grant-in-Aid for Early-Career Scientists from MEXT (17H03542, 17H05739, 17H05761, and 19H04771 to M.S., 19K06674, 19H04956, and 20H05030 to T.Y., and JP20K14364 to Y.T.), Takeda Science Foundation (to M.S. and T.Y.), Cooperative Research of “Network Joint Research Center for Materials and Devices” (to M.S.).

**Author contributions**

M.W. and M.S. conceived and designed the experiments. X.H., C.L., R.T., T.Y., and M.S. performed experiments. M.W. and M.S. acquired, analyzed, and interpreted the data. S.-I. E., M.N., Y.T., and M.S. formulated the mathematical models. M.W. and M.S. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22442-3.

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**Peer review information** *Nature Communications* thanks David Sprinzak, Thomas Vaccari and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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