DeltaA/DeltaD regulate multiple and temporally distinct phases of Notch signaling during dopaminergic neurogenesis in zebrafish

Julia Mahler¹, Alida Filippi¹ and Wolfgang Driever ¹,²,*

(1) Developmental Biology, Institute Biology I, Faculty of Biology, University of Freiburg, Hauptstrasse 1, D-79104 Freiburg, Germany
(2) Freiburg Institute for Advanced Studies, University of Freiburg, Albertstrasse 19, D-79104 Freiburg, Germany

SUPPLEMENTAL MATERIALS

SUPPLEMENTAL FIGURES

Supplementary Figure 1
Inhibition of Notch signaling by the γ-secretase inhibitor DAPT phenocopies the th expression phenotype of mind bomb mutants.
(A-O) Analysis of th expression in 75 hpf embryos treated with DAPT at specific earlier developmental stages. (A-C) WT control; (D-O) WT embryos treated with DAPT at the indicated developmental stages: (D-F) 15-24 hpf, (G-I) 24-36 hpf, (J-L) 36-48 hpf, and (M-O) 48-60 hpf. Compared to WT the treated embryos showed differences in th expression but no severe changes in head/brain morphology.

Supplemental Figure 2
DAPT inhibition of Notch signaling differentially affects catecholaminergic neuronal groups.
Analysis of changes in th expressing cell cluster size in embryos treated during six partially overlapping 10-12 hours time windows with DAPT. Start times of DAPT treatments are indicated at right, top. Analysis was performed for 7 different DA clusters (DC1, DC4, DC3, DC5/6, the telencephalic DA clusters, the preoptic and pretectal cluster) and 2 NA clusters (LC and MO). Each bar represents 100% of the analyzed embryos; the colors reflect the
proportion of the different phenotypic classes as indicated by color code. (*) indicates that in the case of DC3 the increases compared to wildtype were in most cases very small.

**Supplementary Figure 3**

**Stage specific widespread activation of Notch signaling by heat-shock driven overexpression of NICD caused a reduction of th expressing neurons in specific clusters.**

The Notch ICD domain was globally overexpressed in a stage specific manner by heat-shocking embryos from a cross of \textit{HSP70:Gal4} and \textit{Gal4UAS:NotchICD-myc} fish. \textit{th} expression was evaluated by WISH at 72 hpf. Overexpression of NICD earlier than 24 hpf severely disturbed development of the whole embryo including the brain. Therefore, we induced NICD overexpression starting at 24 or 30 hpf, and analyzed the larvae at 72 hpf. (A-C) WT control heat-shocked at 24 hpf. (D-F) overexpression of NICD by heat-shock at 24 hpf led to a reduction of \textit{th} expressing cells in DC1/3/4/5. DC2 was not affected when heat-shocked at 24 hpf (E,F) and 30 hpf (H,I). Following heat-shock at 24 hpf or 30 hpf only a few telencephalic DA neurons and no \textit{th} expression in the pretectum were detectable. (G-I) Following NICD overexpression at 30 hpf, DC5/6 were reduced. Abbreviations: AAC: arch associated cluster; LC: locus coeruleus; Pr: pretectal cluster; sym: sympathetic neurons; TC: telencephalic DA clusters. (C,F,I) dorsal views. Images are z-projections of multiple focal planes to show all CA groups in a single picture; (A,B,D,E,G,H,) lateral views, anterior left. Scale bars: 100 µm (in A for A,D,G; B for B,E,H; C for C,F,I).

**Supplementary Figure 4**

**NICD overexpression at pharyngula stages may cause loss of th expressing cells.**

The Notch ICD domain was globally overexpressed in a stage specific manner by heat-shocking embryos from a cross of \textit{HSP70:Gal4} and \textit{Gal4UAS:NotchICD-myc} fish. At 72 hpf larvae were fixed and \textit{th} expression was evaluated by WISH. Notch overexpression at 36 hpf or 48 hpf affected formation of CNS \textit{th} expressing cells in a highly variable way, ranging from reduction in cell number to complete elimination of \textit{th} expression. An explanation for this variability in phenotype could be that the progeny of heterozygous carrier for the activator and effector line carry different number of transgene copies which result in different levels of NICD overexpression and variable severity of phenotypes. Experimental embryos were individually genotyped for presence of both \textit{HSP70:Gal4} and \textit{Gal4UAS:NotchICD-myc} transgenes, and levels of NICD-myc analyzed by immunohistochemistry for the myc-tag (data not shown). Variability in myc-tag stain intensity supported the hypothesis that variability in
NICD expression may cause the phenotypic variability. (A-C) WT control heat-shocked at 36 hpf. (D-I) Overexpression of NICD by heat-shock at 36 hpf caused in most experimental embryos (D-F) reduction of th expressing neurons in DC1/3/5/6, and TC, complete loss of th expression in Pr, but did not affect the th expression pattern of DC2/4. However, in some experimental embryos (G-I) a complete loss of th expression in the forebrain was detected. (J-L) NICD overexpression at 48 hpf did not affect th expression in DC2/4/5, and 6; however, a reduction of th expressing cells in DC1/3 and TC, and a complete loss of th expression in Pr was observed. No changes in the th expression of AAC and sympathetic ganglia were detectable. (M-O) in some embryos a complete loss of th expression in early differentiating DC2 was observed following heat-shock at 48 hpf. Abbreviations: AAC: arch associated cluster; LC: locus coeruleus; Pr: pretectal cluster; sym: sympathetic neurons; TC: telencephalic DA clusters. (A,D,G,J,M) lateral views; (B,C,E,F,H,I,K,L,N,O) dorsal views, anterior to the left. Images are z-projections of multiple focal planes to show all CA groups in a single picture. Scale bars: 100 µm.

**Supplementary Figure 5**

**NICD overexpression between 36 and 48 hpf affects expression of the DA specification and differentiation factors sim1a and otpa and causes upregulation of the glial marker gfap.**

The Notch ICD domain was globally overexpressed in a stage specific manner by heat-shock treatment of embryos from a cross of HSP70:Gal4 and Gal4UAS:NotchICD-myc fish. (A-H) otpa, sim1a and gfap expression was analyzed by WISH at 72 hpf. (A-C) control embryos heat-shocked at 36 hpf. The expression of the DA specification and differentiation genes otpa (A,D,G) and sim1a (B,E,H) was downregulated following heat-shock induced NICD overexpression at 36 hpf (D,E) and 48 hpf (G,H). NICD overexpression at 48 hpf had a much less severe effect on otpa expression. The expression of the radial glia marker gfap was upregulated in the rhombencephalon, whereas the MHB expression domain was lost after heat-shock at 36 hpf (F). Images are lateral views, anterior to the left. Scale bar: 100 µm.

**Supplementary Figure 6**

**Effect of NICD overexpression on formation of isl1 expressing neurons.**

As a control for efficient Notch-ICD overexpression we analyzed isl1 expression in heat-shocked embryos from a cross of HSP70:Gal4 and Gal4UAS:NotchICD-myc fish. (A-J)
Analysis of *islet1* expression at 72 hpf following heat-shocks at the stages indicated at left. (A,B) WT control heat-shocked at 24 hpf. (C-J) *isl1* expression pattern in embryos transgenic for both hsp70:Gal4 and UAS:NICD was changed following heat-shock at 24 hpf (C,D), 30 hpf (E,F), 36 hpf (G,H), and 48 hpf (I,J). While early NICD overexpression causes massive loss of *isl1* expressing neurons in hindbrain and retina, diencephalic clusters appear increased in stain intensity. Heat-shocks as late as 48 hpf have little effect on *isl1* expressing neurons. (A,C,E,G,I) lateral views, (B,D,F,H,J) dorsal views, anterior left. Scale bars: 100 µm.

**Supplementary Figure 7**

*deltaC and jagged1a/b are not expressed in the ventral diencephalon at 24 hpf.*

Expression analysis by WISH for *dlC* (A,B) at 24 hpf, *jag1a* (C,D), and *jag1b* (E,F) at 18 hpf in the forebrain of WT zebrafish embryos. These three Notch ligands were not expressed in the diencephalon at the analyzed developmental stages. (A,C,E) dorsal views, (B,D,F) lateral views, anterior left. Scale bars: 100 µm.

**Supplementary Figure 8**

*Loss-of-function analysis of jagged2 and dB indicates no role in formation of th expressing neurons in the zebrafish forebrain.*

(A-L) *th* expression was analyzed at 72 hpf to reveal DA neurons in ventral diencephalon. (A-H) Changes in *th* expression pattern were not detectable in embryos injected at 1 cell stage with an ATG MO against *jagged2a* (C,D compare to WT A,B) or in *jagged2ha3425* mutants (G,H compare to WT E,F). (I-L) Knock-down of *dlB* by ATG MO injection caused no differences of *th* expression pattern in injected embryos (K,L) compared to non-injected siblings (I,J). (A,C,E,G,I,K) dorsal views, images are z-projections of multiple focal planes to show all CA groups in a single image; (B,D,F,H,J,L) lateral views, anterior left. Scale bars: 100 µm.

**Supplementary Figure 9**

*Proliferation and general precursor markers are not affected in *dla*hi781 mutants.*

*pcna* (A,B), *sox2* (C,D) and *ngn1* (E,F) expression patterns (green) were analyzed by fluorescent in situ hybridization in WT siblings (A,C,E) and *dla*hi781 mutant embryos (B,D,F). DA neurons were visualized by anti-TH immunohistochemistry (red). No significant changes were observed in the expression of these markers between WT and mutant siblings at the stages analyzed.
**Supplementary Figure 10**

**Birthdating analysis in dla^{hi781} mutant embryos.**

WT and dla^{hi781} mutant siblings were labeled with EdU at 8 (A-B''') and 24 (C-D''') hpf, and analyzed at 72 hpf by anti-TH immunohistochemistry (red) and EdU Click-It Alexa488 label (green). No significant differences in EdU incorporation were observed between control and mutant embryos, suggesting that proliferation rate is not affected by lack of DeltaA activity, and that the supernumerary DC2 neurons observed in dla^{hi781} mutants do not derive from additional rounds of cell division.

**Supplementary Figure 11**

**Expression of DA precursor and differentiation transcription factors otpa and sim1a is increased in mind bomb mutants.**

(A-H) Analysis of changes in expression of otpa and sim1a, which are expressed in ventral diencephalic posterior tubercular DA precursors and differentiating neurons. At 24 hpf, upregulation and expansion of otpa expression domains were observed in the diencephalon of mib^{ma52b} mutants (B,D) when compared to WT siblings (A,C); sim1a ventral diencephalic domain was also expanded and upregulated in mib^{ma52b} mutants (F,H, arrows) compared to WT siblings (E,G), while a significant reduction of sim1a expression was observed in the mesencephalon (F, arrowhead). DC, diencephalon. (A,B,E,F) lateral views, (C,D,G,H,) dorsal views, anterior left. Scale bar: 100 µm.
Mahler et al., Supplemental Figure 2
Mahler et al., Supplemental Figure 3
Mahler et al., Supplemental Figure 4
expression at 72 hpf

<table>
<thead>
<tr>
<th>otpa</th>
<th>sim1a</th>
<th>gfap</th>
</tr>
</thead>
</table>

Stage starting heat-shock

<table>
<thead>
<tr>
<th>36 hpf, wt control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>36 hpf, UAS:NICD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>F</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>48 hpf, UAS:NICD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
</tr>
<tr>
<td>H</td>
</tr>
</tbody>
</table>

Mahler et al., Supplemental Figure 5
*islet1* expression at 72 hpf

**Stages starting heat-shock**

- **24 hpf**
- **30 hpf**
- **36 hpf**
- **48 hpf**

---

Mahler et al., Supplemental Figure 6
Mahler et al., Supplemental Figure 7
Mahler et al., Supplemental Figure 8
Mahler et al., Supplemental Figure 9
Mahler et al., Supplemental Figure 10
Mahler et al., Supplemental Figure 11