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Function of the Notch/Delta Pathway in Ophthalmic Trigeminal Placode Development

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FUNCTION OF THE NOTCH/DELTA PATHWAY IN OPHTHALMIC TRIGEMINAL PLACODE DEVELOPMENT

By

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the dissertation of Matthew K. Ball in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

FUNCTION OF THE NOTCH/DELTA PATHWAY IN OPHTHALMIC TRIGEMINAL PLACODE DEVELOPMENT

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Master of Science

The ophthalmic trigeminal placode (opV) is the birth place of one cell type of sensory neurons contributing to the trigeminal ganglion. Signals from the neural tube induce placodal identity within the surface ectoderm. Specified opV placode cells then up-regulate neuron differentiation markers and migrate to the ganglion. Several molecular pathways have been shown to act in opV placode cell development. Despite this, signals that specify individual neurons from within the opV placode remain unknown. However, it is known that components of the Notch signaling pathway are expressed in the opV placode. I tested the role of Notch signaling in opV placode development by separately inhibiting and over-activating the pathway. Using DAPT, an inhibitor of gamma-secretase, I inhibited Notch signaling in 13-15 somite stage chick embryo heads. Attenuated Notch signaling caused increased neuronal differentiation of opV cells at 13-15 somites. I also observed an increase in migratory opV placode (Pax3+) cells in the

mesenchyme and expression of neuronal marker Islet1 in the ectoderm. Further, I activated Notch signaling by misexpressing the Notch intracellular domain (NICD) by in ovo electroporation of 10-12 somite stage chick embryos. This resulted in Pax3+ targeted cells failing to differentiate and remain instead in the ectoderm. Thus, Notch/Delta signaling plays an important role in selecting ophthalmic trigeminal cells to differentiate and migrate to the trigeminal ganglion.

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Introduction

Cranial placodes are areas of head ectoderm (Fig. 1) that contribute to sensory organs and cranial ganglia. For example, the otic placode forms the inner ear, the geniculate placode forms the facial nerve, and two trigeminal placodes form sensory neurons of the trigeminal ganglion including its three branches: the maxillary, mandibular and ophthalmic. The ophthalmic branch conveys the sense of touch, temperature, proprioception and pain to the forehead, upper nose and eye. Neurons in the ophthalmic branch come from the ophthalmic trigeminal placode (opV), which is marked by the expression of Pax3.

The opV placode provides a relatively simple system for sensory neurogenesis, which is amenable to cell-autonomous manipulation by electroporation and non cell-autonomous manipulation through tissue culture. Recently, there has been interest in elucidating the pathways controlling opV specification and fate determination. Developmental pathways shown to affect the opV fate include Wnt (Lassiter et al. 2007), FGF (Lassiter et al. 2009), PDGF (McCabe, Bronner-Fraser 2008) and Shh (Fedtsova, Perris & Turner 2003). As an example, the canonical Wnt pathway was shown to be necessary, but not sufficient to induce the opV placodal fate (Lassiter et al. 2007). Molecular components of these pathways were initially observed in opV through in situ hybridization and immunohistochemistry. The presence of these pathway molecules led to experiments aimed at understanding their functions. Consistent with this mode of discovery, key components of the Notch/Delta pathway reside in the opV placode (McCabe et al. 2004, Begbie, Ballivet & Graham 2002).

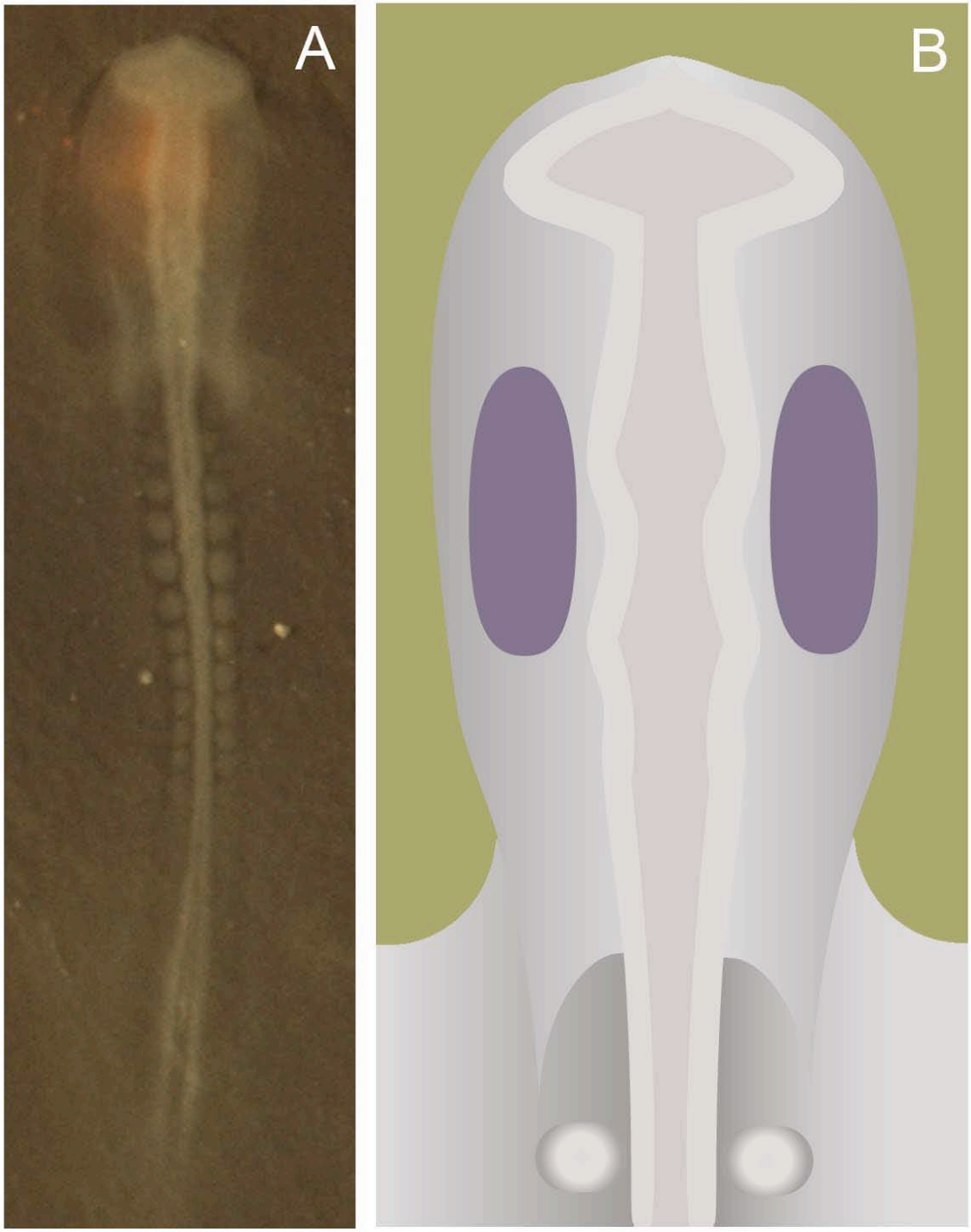

Figure 1. Location of the Trigeminal Placode
(A) 12 somite stage chick embryo. (B) 12 somite stage chick embryo head.The location of the trigeminal placode is highlighted in purple.

Yet, despite the presence of the Notch/Delta pathway and known functions of this pathway in neurogenesis, its function in the opV placode remains unknown.

Notch is a single pass transmembrane receptor which is bound by ligands Delta and Serrate (also known as Jagged) on adjacent cells. Once ligand binding occurs, a series of protein cleavages (with the final cleavage being made by gamma-secretase) causes the Notch intracellular domain (NICD) to be released into the cytosol. Cleaved NICD then functions as a transcription factor by entering the nucleus and activating transcription of target genes (reviewed in Bray 2006). NICD activates gene transcription when it binds to CSL and Mastermind on the target genes' promoters (Fig. 2). The Notch/Delta pathway has different functions depending on when and where it is activated in development. The Notch/Delta pathway is important to many developmental processes including somite formation (Pourquie 2001), neural progenitor maintainance (Lasky, Wu 2005), hematopoesis (Cheung et al. 2006) and angiogenesis (Karamysheva 2008). It is also active in the development of the cardiac ventricals, pancreas, bone, blood, and has a prominent role in certain types of cancer (Fiúza, Arias 2007, Bolós, Grego-Bessa & de la Pompa, José Luis 2007, Weng et al. 2003). Generally, Notch signaling acts to *maintain stem cells*, *laterally inhibit* cell fate, and *laterally induce boundary formation*.

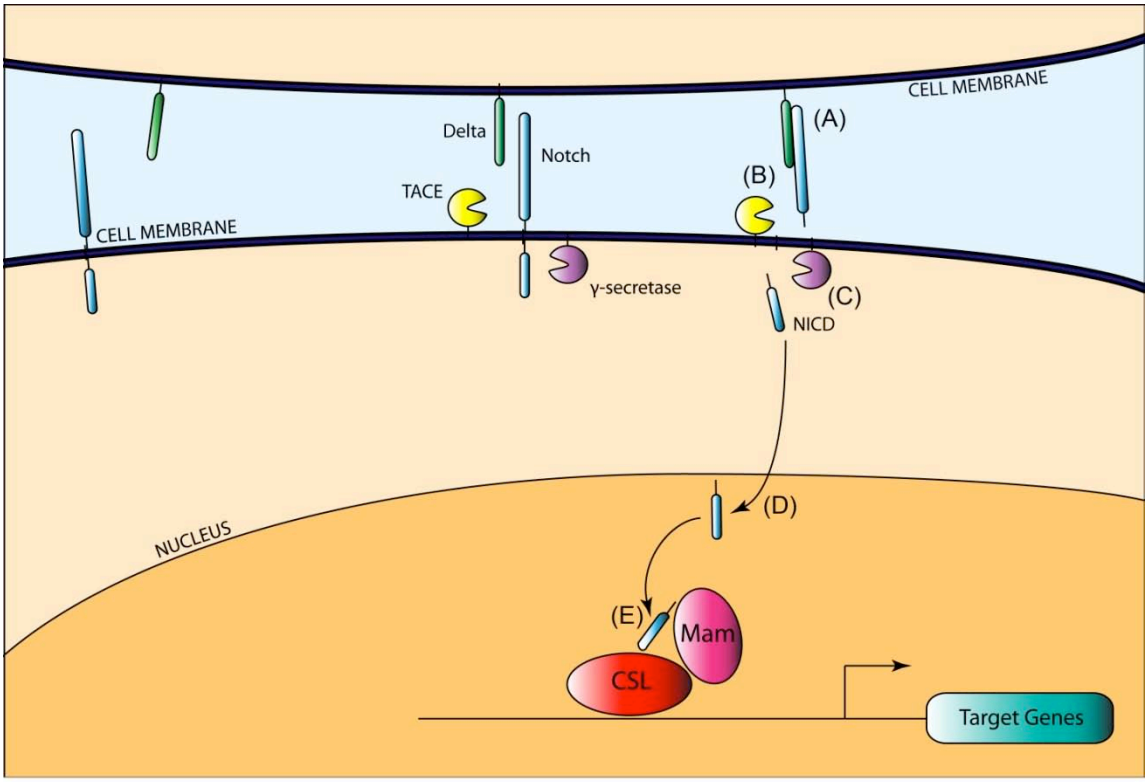

Figure 2. The Notch/Delta Pathway
(A) Delta on one cell binds to Notch on an adjacent cell.
(B) The Notch extracellular domain is cleaved by TACE.
(C) The Notch intracellular domain (NICD) is cleaved by gamma-secretase.
(D) NICD translocates to the nucleus.
(E) NICD binds to CSL and Mastermind (Mam) and activates target gene transcription.

Maintenance of Progenitor Cells

Activated Notch signaling is known to maintain progenitor stem cells (Akai, Halley & Storey 2005). Continual Notch signalling between cells keeps the stem cells in an undifferentiated state until the cells undergo lateral inhibition (discussed below). When stem cell maintenance is perturbed, the differentiation of progenitor cells is *initially* observed as an increase in neurons compared to wild type, but *eventually* the early differentiation of stem cells results in fewer total cells. An example of this is seen in Notch1 and RBPjK (known as CSL in chick) knockout mice, which show premature differention of neurons causing the depletion of neural progenitors (Bolós, Grego-Bessa & de la Pompa, José Luis 2007, Yoon, Gaiano 2005). An example of an increase in neurons following Notch inhibition has been observed with the gamma-secretase inhibitor, DAPT (Abelló et al. 2007, Daudet, Ariza-McNaughton & Lewis 2007, Nelson et al. 2007). Gamma-secretase functions to cleave NICD from the Notch receptor, thus allowing it to enter the nucleus and activate Notch signaling (Fig. 2). Therefore, when gamma-secretase is inhibited by DAPT, Notch signaling is inhibited and the stem cells prematurely differentiate. Maintainance of stem cells usually preceeds cell fate determination through lateral inhibition.

Lateral Inhibition

Perhaps the most well known mechanism of neural progenitor cell selection is lateral inhibition. The classical view of lateral inhibtion involves a field of cells with the same developmental potential; that is, all cells express both Notch and Delta. Cells in the field expressing Delta activate Notch signaling in cells lateral to them and inhibit their differentiation into neurons (hence the name, lateral inhibition). When a dominate-active

Delta was expressed through a retroviral vector into the chick eye, it activated Notch signaling in all adjacent cells, thus inhibiting neuronal differentiation. When a dominate-negative Delta was expressed the opposite result was achieved and many neurons were observed. This was due to Notch signaling not being activated in these cells (Lewis 1998). Neuronal inhibtion is accomplished through Hes1 activation. When Hes1 is expressed, it binds and inhibits Ngn2 protein. Hes1 also binds and inhibits the promoter of Ngn2, thus stopping its transcription. In cells that do not have Notch signaling activated Delta expression increases along with Ngn2 and the cells are committed to a neuronal fate (Kageyama et al. 2005).

Boundary Formation

Notch signalling is also necessary in forming boundaries between compartments (also called lateral induction). In boundary formation Hes genes (such as Hes1, Hes3 and Hes5) are expressed at persistently high levels. This keeps cells from expressing proneural genes and becoming neurons. In a Hes1;Hes3;Hes5 knockout mouse proneural genes are expressed in boundary areas, showing that the boundaries of these embryos have become disrupted (Baek et al. 2006). Though this is an important function of Notch signaling in the whole embryo, it is unknown whether any boundaries form in the opV placode.

Notch/Delta pathway genes are known to be expressed at a time consistent with onset of neurogenesis in the opV placode (Begbie, Ballivet & Graham 2002) and the Notch/Delta pathway is known to be involved in neurogenesis. I inhibited the Notch/Delta pathway in the ophthalmic trigeminal placode using DAPT and found that the number of neurons increased. I also over-activated the Notch/Delta pathway using electroporation of NICD

and found that the number of neurons decreased. Through these experiments we determined that the Notch/Delta pathway plays an important role in selecting neurons in the ophthalmic trigeminal placode. These experiments lay the foundation to further understand the mechanism by which the Notch/Delta pathway acts in the opV placode.

Materials and Methods

Tissue culture experiments

Fertilized chicken (Gallus gallus) eggs bought from local farms were incubated in a humidified incubator at 38°C until the 13-15 somite stage. Embryos were collected into 1X PBS and cleaned of extraembryonic tissues, then placed in complete medium (~88% Dulbecco's Modified Eagle Medium, ~9% fetal bovine serum (FBS), ~2% chick serum (CS), ~1% penstrep) on ice. Embryos were rinsed in no serum media (~99% Dulbecco's Modified Eagle Medium, ~1% penstrep) three times to remove FBS and CS. The head regions were removed at the level of the otic placode. Heads were placed in collagen matrix gels as described with modifications as described below (Abelló et al. 2007; Groves, Bronner-Fraser 2000). Briefly, collagen gel solution (90µl collagen type I, 10 µl 10X DMEM with the pH adjusted to 7.5 with 7.5% Sodium Bicarbonate) was pipetted (20 µl) into 12 well culture dishes and allowed to set. Head regions were embedded in the collagen solution, dorsal side up and 20 µl of additional collagen solution was added on top of the embryo heads. After additional collagen was set, one milliliter of DMEM with N2 supplement (GIBCO) was added to each dish along with either DAPT (100µM, Abelló et al. 2007) dissolved in dimethylsulfoxide (DMSO) or DMSO alone, as a control. Cultures were grown at 37° and 5% CO_2 for 24 hrs. After culture, the heads were fixed in 4% formaldehyde for 2-4 hours, removed from surrounding collagen and prepared for cryosection.

N-(3, 5-difluorophenacetyl-L-alanyl)-S-phenylglycine t-butyl ester (known as DAPT, obtained from Calbiochem) was used to inhibit Notch signaling. It is a small hydrophobic

molecule that inhibits gamma-secretase. Therefore, NICD is not cleaved from the Notch receptor, so Notch signaling is inhibited.

In ovo electroporation

Eggs were incubated until the 10-12 somite stage. A window was cut in the top of the egg to allow manipulation of the embryo and 1:10 India ink/PBS was applied under the hypoblast to visualize the embryo. A 3:20 dilution of fast green to plasmid DNA (~5 µg/µl) was loaded into micropipetes and pipeted near the opV placode.

Electrodes were placed above and below the head and seven, 10 ms pulses at 10 -18 volts was applied through the opV placode (BTX 820 electroporator from Gentronics). Application of PBS with 1:1000 penstrep was added to prevent dehydration and infection. Egg windows were sealed with tape and allowed to incubate an additional 30 hours. Embryos were then collected into 4% formaldehyde and electroporation targeting was observed. Embryos were excluded that were obviously dead, severly deformed, or which were not targeted. Remaining embryos were prepared for cryosection.

Constructs used were pCIG-NICD-GFP (refered to as NICD) and pCIG-GFP (refered to as pCIG). pCIG expresses a nuclear GFP, which assisted in discerning the separation between cells in fluorescent images. The NICD used is a mouse sequence cloned into the pCIG vector. This construct cell-autonomously activates Notch signling in chicken as well as mouse. An empty pCIG vector was used as a control. NICD and pCIG electroporation constructs were a kind gift of Dr. Andrew McMahon.

Immunohistochemistry of cryosections

Embryos were equalized in sucrose, embedded in 300 Bloom gelatin, and snap frozen with liquid nitrogen. Frozen gelatin blocks were sectioned at 12µm with a cryostat and mounted on Superfrost® Plus glass slides. Gelatin was removed by placing the embryos in PBS for 15 minutes at 37°C. Primary antibodies Pax3 (at 1:200 or 1:1000, mouse IgG2a, (Baker et al. 1999) and Islet1 (at 1:200, mouse IgG2b, Developmental Studies Hybridoma Bank) were diluted in PBS/BSA/Tween and applied to the slides. After incubation overnight at 4°C primary antibody was washed away. Secondary antibodies (Molecular Probes) Alexa^{546}-conjugated goat anti-mouse IgG2a diluted 1:1000 and Alexa^{488}- or Alexa^{633}- conjugated goat anti-mouse IgG2b diluted 1:200 and 1:175 respectively in PBS, 0.1% Bovine Serum Albumen, 0.1% Tween were applied and allowed to incubate for 1-2hrs at 25°C. Slides were washed to remove antibody, stained with DAPI (to control for apoptosis), and coverslipped. Fluorescent images were taken at 20X using an Olympus BX61 fluorescent microscope. Embryo sections and images were kept in order to determine the middle of the placode.

Quantitative Analysis

Five random sections through the middle of the opV placode were counted for each placode using Olympus Microsuite (Lassiter et al. 2007, Lassiter et al. 2009). Pax3, GFP and Islet1 postive cells were defined by minimum color thresholds. Due to the differences between pictures, approproiate theresholds were determined based on the anatomy of the placode and the normal wild type expression pattern of these genes.

In tissue culture samples, Pax3+, Islet1+ and Pax3/Islet1 cells were counted in the ectoderm and the mesenchyme for both DAPT and DMSO. For electroporations, GFP+, Pax3+, Pax3/GFP, and Pax3/GFP/Islet1 cells were counted in the ectoderm and the mesenchyme for both NICD and pCIG. Student's t-test was performed and p-values determined to test for significance.

Results

Inhibition of Notch signaling causes differentiation of the placode

In order to test the function of the Notch/Delta pathway in the trigeminal ganglia we decided to inhibit Notch signaling using the gamma-secretase inhibitor, DAPT. DAPT has been successfully used by other researchers to inhibit the Notch pathway of chick embryos in the retina and otic placode (Nelson et al. 2007, Abelló et al. 2007, Daudet, Ariza-McNaughton & Lewis 2007). Whole heads of 13-15 somite stage chick embryos were cultured in DAPT or DMSO (control). According to one report it takes only 6 hours before DAPT commits the first cells to a neuronal fate in the chick retina, but takes 24 hours for all regions of the retina to be committed (Nelson et al. 2007). An incubation time of 24 hours was also used to commit neural progenitor cells in the otic placode (Daudet, Ariza-McNaughton & Lewis 2007). I cultured 13-15 somite stage embryos for 24 hours allowing ample time to commit progenitor cells residing in the opV placode to a neuronal fate. The opV placode of wild type chick embryos are known to have continual neuronal differentiation throughout this culture period.

In these assays, molecular makers Pax3 and Islet1 were analyzed to determine the identity of the opV cells. Neurons contained in the ophthalmic branch of the trigeminal nerve come from cells of the ophthalmic trigeminal placode (opV) and the cranial neural crest (D'Amico-Martel, Noden 1983). OpV placode cells express significantly more Pax3 than neural crest in the opV ganglion, allowing us to differentiate between the two. Neurons in the maxillary and mandibular trigeminal branches come from the maxillo-mandibular trigeminal placode (mmV), which does not express Pax3. This makes Pax3 an important identity marker for the opV placode, allowing us to differentiate it from the

mmV placode. Islet1 is expressed in differentiating cells of both the opV and mmV placodes. Thus, cells that are Pax3 and Islet1 positive are differentiating opV placode cells, whereas cells that are Pax3 negative and Islet1 positive are differentiating mmV placode cells.

Compared to DMSO controls (n=17 placodes), the DAPT treated (n=17 placodes) embryos did not show a significant increase in the number of Pax3 positive cells in the ectoderm (Fig. 4A). This suggests that the size of the placode did not increase, but remained constant between DAPT and DMSO. This is in contrast to a significantly increased number of Pax3 positive cells in the mesenchyme of DAPT treated heads (75.88 cells/placode to 188.12 cells/placode; Fig. 4C, p=0.000071). Increased Pax3 expression in the mesenchyme almost always colocalized with Islet1 expression. This suggests that these mesenchymal Pax3 positive cells had begun the process of differentiation (Table 1).

There was also a distinct and significant increase in differentiation of opV placodal neurons marked by colocalization of Pax3/Islet1 in both the ectoderm and the mesenchyme (p=0.00000009 and p=0.000069, respectively, Fig. 4B, D). The fact that Islet1 was so strongly up-regulated in the ectoderm is interesting, because such mass differentiation in the ectoderm has not previously been reported (Fig. 3). It is unclear why the Pax3/Islet1 positive cells in the ectoderm did not immediately delaminate, but this finding does suggest that differentiation may not be coupled to delamination in these cells.

Another important observation was that the mmV placode also prematurely differentiated like the opV placode. Islet1 cells could be seen in the ectoderm of this placode too, which is not seen in wild type embryos (Fig. 5).

Statistics on the Tissue Culture of DMSO vs. DAPT			
DMSO (control) Sample Size = 17	Ectoderm		
DAPT Sample Size = 17	Total Pax3	Total Islet1	Pax3/Islet1
DMSO (control) Mean	121.18	10.88	9.06
DAPT Mean	129.24	149.71	119.59
DMSO (control) SEM (+/-)	10.62	1.29	1.18
DAPT SEM (+/-)	12.97	14.75	11.96
p-values	0.637	0.00000007	0.00000009
Result, α=0.05	Do Not Reject Ho	Reject Ho	Reject Ho

DMSO (control) Sample Size = 17	Mesenchyme		
DAPT Sample Size = 17	Total Pax3	Total Islet1	Pax3/Islet1
DMSO (control) Mean	75.88	80.06	64.94
DAPT Mean	188.12	253.88	171.12
DMSO (control) SEM (+/-)	7.84	8.57	6.71
DAPT SEM (+/-)	19.64	23.14	18.79
p-values	0.000071	0.000003	0.000069
Result, α=0.05	Reject Ho	Reject Ho	Reject Ho

Table 1. Tissue Culture Statistics

"Total Pax3" refers to all the Pax3 positive cells present in either the ectoderm or the mesenchyme. "Total Islet1" refers to all the Islet1 positive cells present in either the ectoderm or the mesenchyme. "Pax3/Islet1" refers to the colocalization of Pax3 and Islet1 cells in either the ectoderm or the mesenchyme. Standard Error of the Means (SEM); p-values are based on Student's t-test.

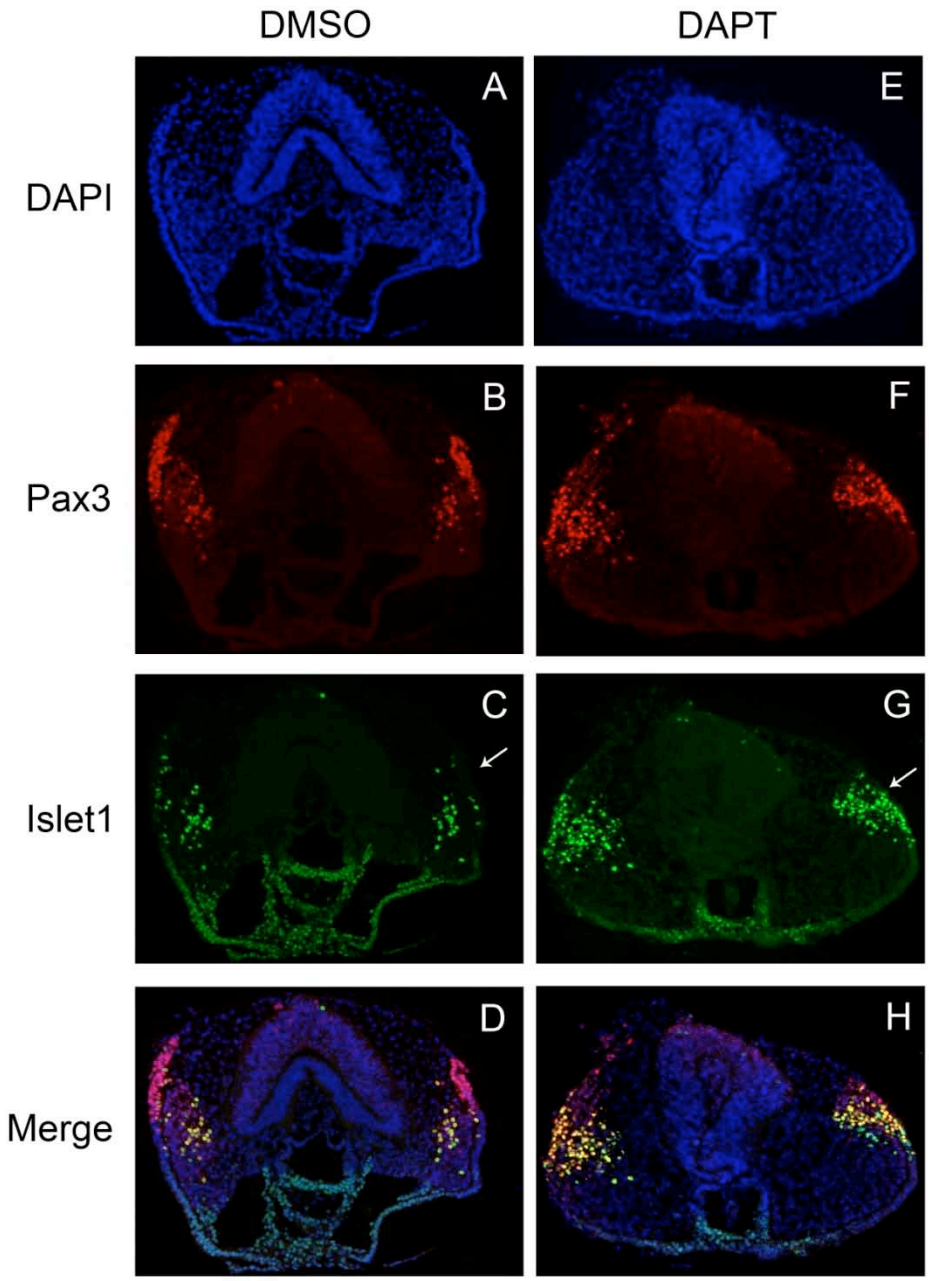

Figure 3. Inhibition of Notch signaling causes premature neuronal differentiation
Transverse sections through 13-15ss chick embryo heads cultured for 24 hours. (A-D) DMSO (control) treated embryo; (E-H) DAPT treated embryo. (A,E) DAPI (blue) nuclear stain to control for apoptosis. (B,F) Pax3 (red) shows an increased population of Pax3 cells in DAPT treated embryo over DMSO control. (C,G) Islet1 (green) expression in the opV placodes. DAPT treated placodes show an increase in Islet1 positive cells (arrows). There is also increased Islet1 expression in the ganglion. (D,H) Merge of DAPI (blue), Pax3 (red) and Islet1 (green). Colocalization of Pax3 and Islet1 is seen as yellow.

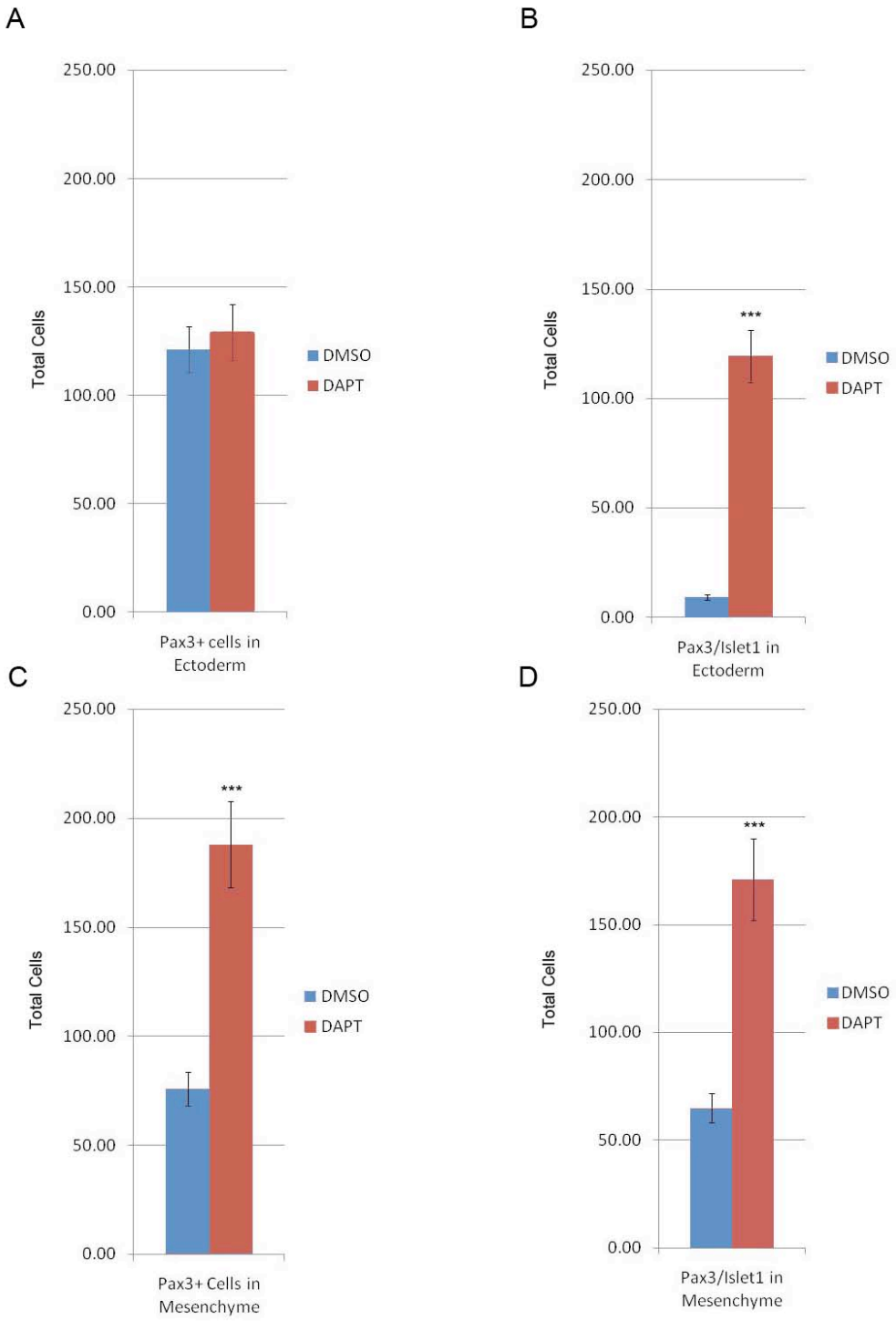

Figure 4. Tissue Culture of 13-15ss chick embryos incubated for 24hrs
(A) Pax3 positive cells in the ectoderm.
(B) Colocalization of Pax3 and Islet1 in the ectoderm.
(C) Pax3 positive cells in the mesenchyme.
(D) Colocalization of Pax3 and Islet1 in the mesenchyme.
Error bars represent Standard Error of the Mean (SEM).
(*) p-value < 0.05; (**) p-value < 0.001; (***) p-value < 0.0001

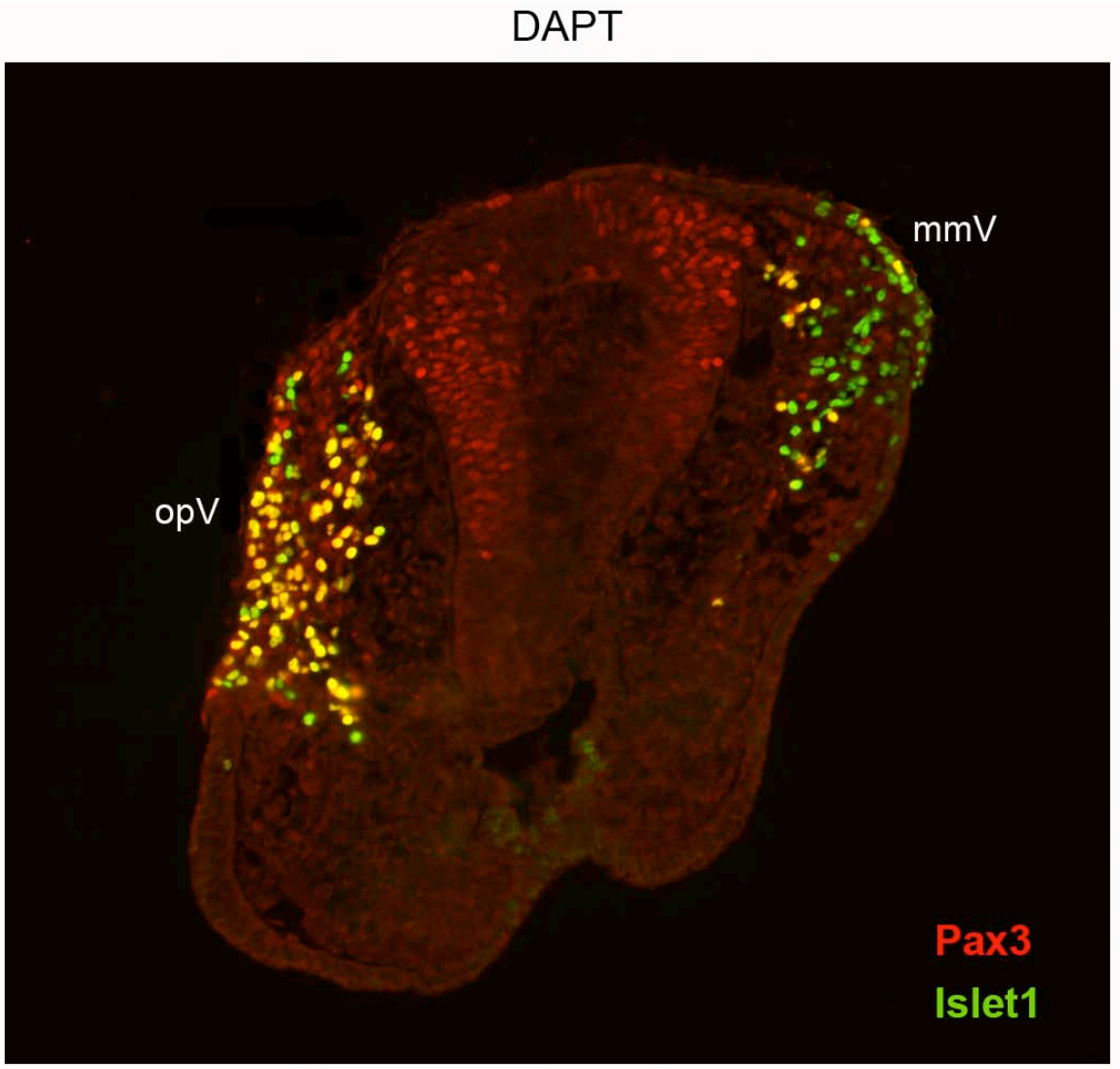

Figure 5. DAPT causes differentiation in mmV placode
DAPT causes the mmV placode to express Islet1 similar to the opV placode. For comparison, the opV and mmV placodes can both be seen in this section due to the angle of section.

Activation of Notch signaling inhibits differentiation of the placode

I also activated Notch signaling in the opV placode using the electroporation construct pCIG-NICD (herein referred to as NICD). The construct I used activates Notch signaling by generating the Notch intracellular domain (NICD) inside targeted cells. NICD translocates to the nucleus, binds to CSL and recruits Mastermind to the promoters of target genes. Hes1 is a target of Notch signaling and is likely to be up-regulated in NICD targeted cells. Hes1 then binds to Ngn2 and its promoter to inhibit Ngn2 activation, thus causing neurogenesis to be inhibited and the cells to remain in the ectoderm. This phenotype is observed in our NICD electroporated embryos (Fig. 6 and 7). I chose to electroporate the embryos at the 10-12 somite stage and incubate the eggs for 30 hours to obtain similar endpoint stages to the DAPT/DMSO experiments. Neurogenesis was inhibited with a 191 fold reduction per placode (pCIG, 118.5 cells to NICD 0.62 cells, Table 2) with a p-value of 0.00038.

Pax3 expression in the ectoderm and the mesenchyme was also significantly reduced following Notch activation with NICD (Fig 8A, C; Table 2). This might be expected considering an increase in Pax3 expression was observed when I inhibited the Notch pathway using DAPT. To control for a difference in targeting a t-test was done to find if there was a difference in electroporation targeting. The targeting in the ectoderm between NICD and pCIG is not statistically different ($p=0.651$, Table 2). This would suggest that the reduction in total Pax3 in the mesenchyme did not come from a bias in electroporation.

Statistics on the Electroporation of pCIG vs. NICD				
pCIG (control) Sample Size = 10	Ectoderm			
NICD Sample Size = 13	Total Pax3	Total GFP	Pax3/GFP	Pax3/GFP/Isl1
pCIG (control) Mean	71.70	115.90	45.80	5.50
NICD Mean	47.92	104.62	14.15	3.38
pCIG (control) SEM (+/-)	12.93	22.50	10.53	1.49
NICD SEM (+/-)	5.65	8.87	2.77	0.92
p-values	0.126	0.651	0.017	0.258
Result, α=0.05	Do Not Reject Ho	Do Not Reject Ho	Reject Ho	Do Not Reject Ho

pCIG (control) Sample Size = 10	Mesenchyme			
NICD Sample Size = 13	Total Pax3	Total GFP	Pax3/GFP	Pax3/GFP/Isl1
pCIG (control) Mean	281.60	160.30	132.30	118.50
NICD Mean	103.92	12.31	2.23	0.62
pCIG (control) SEM (+/-)	39.42	26.29	22.75	21.45
NICD SEM (+/-)	20.11	2.56	0.57	0.24
p-values	0.00304	0.00033	0.00029	0.00038
Result, α=0.05	Reject Ho	Reject Ho	Reject Ho	Reject Ho

Table 2. Electoporation Statistics

"Total Pax3" refers to all the Pax3 positive cells present in either the ectoderm or the mesenchyme. "Total GFP" refers to all the GFP positive cells present in either the ectoderm or the mesenchye. "Pax3/GFP" refers to the colocalization of Pax3 and GFP cells in either the ectoderm or the mesenchyme. "Pax3/GFP/Isl1" refers to the colocalization of Pax3, GFP and Islet1 cells in either the ectoderm or the mesenchyme. Standard Error of the Means (SEM); p-values are based on Student's t-test.

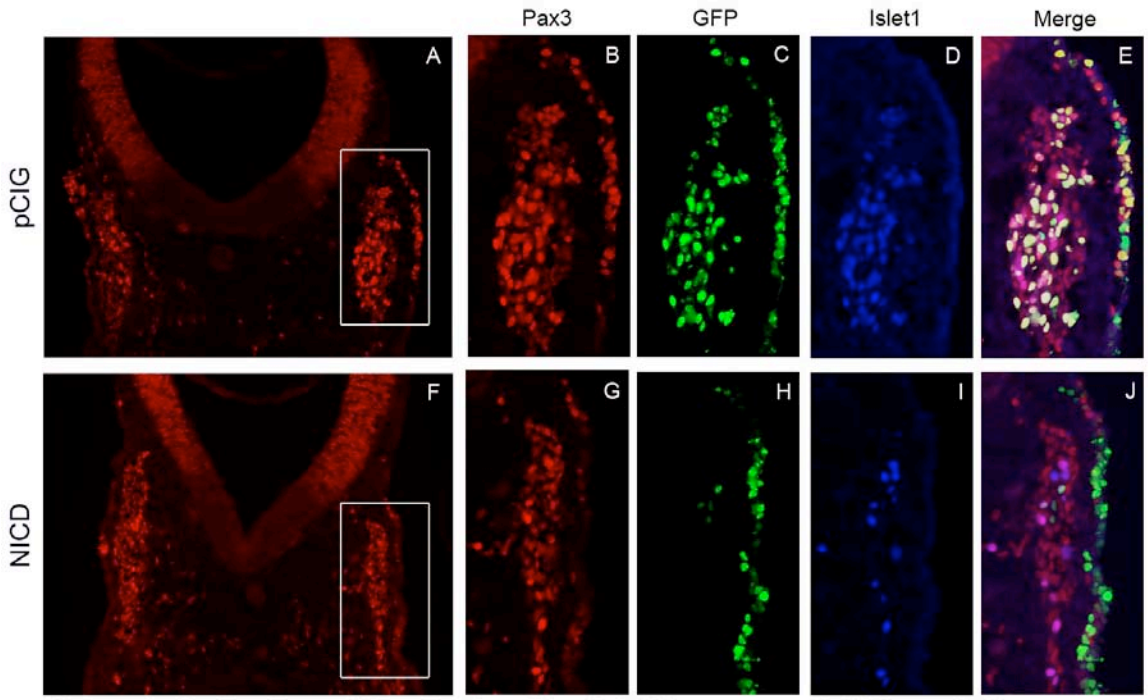

Figure 6. Activation of Notch signaling inhibits neuronal differentiation
Transverse sections through chick embryos electroporated at 10-12ss and incubated for 30hrs. (A-E) pCIG (control) electroporation; (F-J) NICD electroporation; (A-B, F-G) Pax3 expression in the opV placode. Decreased expression of Pax3 is seen in the placode and ganglion of NICD electroporated embryos. (C,H) GFP targeting of the opV placode. pCIG targeted cells are able to delaminate from the placode and form a ganglion. NICD targeted cells remain in the ectoderm. (D,I) Islet1 expression in the opV ganglion. Note that fewer Islet1 expressing cells reside in the NICD targeted placode due to decreased delamination of opV placode cells. (E, J) Merge of Pax3 (red), GFP (green), and Islet1 (blue) images. Colocalization of Pax3 and GFP is yellow and is seen in the ectoderm of pCIG, but not NICD embryos. Colocalization of Pax3, GFP and Islet1 is white and is seen most abundantly in the pCIG targeted ganglia and is not seen in NICD targeted ganglia. Colocalization of Pax3 and Islet1 is magenta and is seen in both pCIG and NICD ganglia, because these cells are untargeted.

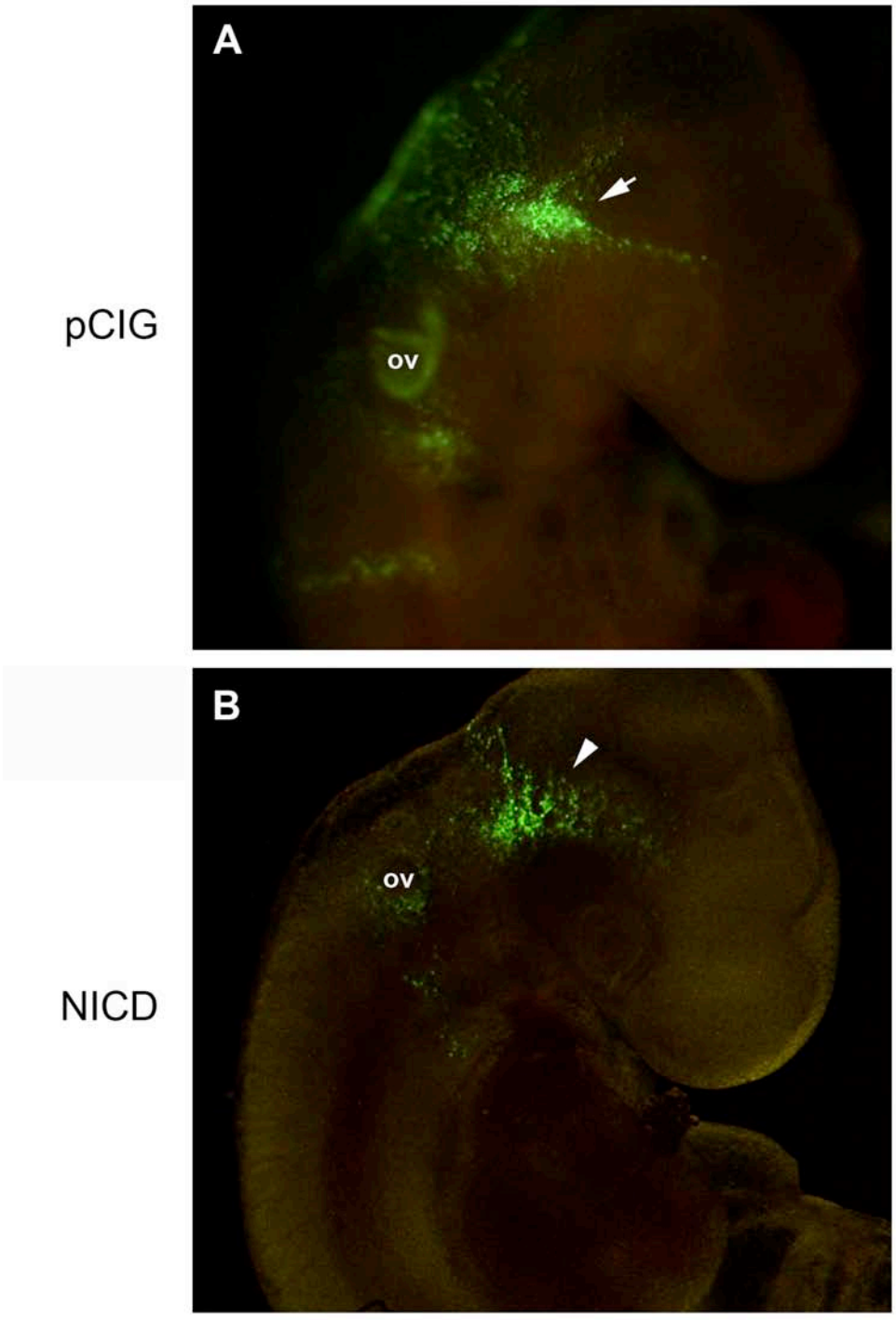

Figure 7. Whole-mount electroporation targeting
Embryos collected at 30-32ss after electroporation at 10-12ss and incubation for 30 hours. (A) pCIG (control) targeted embryo. Targeted cells are observed in the opV ganglion (arrow). (B) NICD targeted embryo. Targeted cells remain in the ectoderm and do not form a distinct ganglion (arrowhead). (ov) otic vessicle.

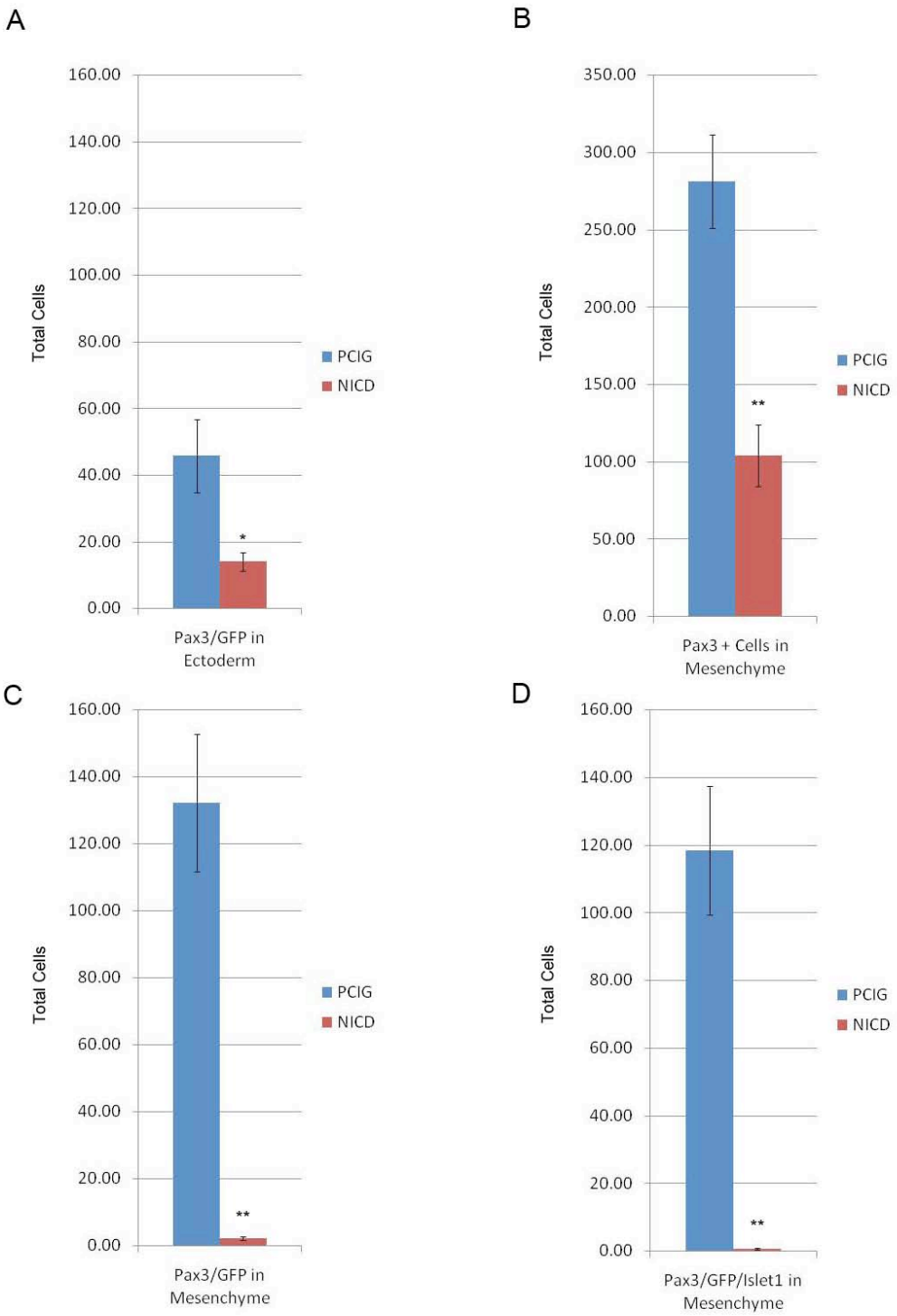

Figure 8. Electroporation of 10-12ss chick embryos incubated for 30hrs
(A) Colocalization of Pax3 and GFP in the ectoderm.
(B) Pax3 positive cells in the mesenchyme.
(C) Colocalization of Pax3 and GFP in the mesenchyme.
(D) Colocalization of Pax3, GFP and Islet1 in the mesenchyme.
Error bars represent Standard Error of the Mean (SEM).
(*) p-value < 0.05; (**) p-value < 0.001; (***) p-value < 0.0001

Discussion

Because of the close developmental timing of Notch pathway gene expression (Begbie, Ballivet & Graham 2002) and the known function of the Notch pathway in neurogenesis, I hypothesized that Notch signaling was involved in the neurogenesis of the opV placode. I used the gamma-secretase inhibitor, DAPT to elicit neuronal differentiation in opV placode cells. The neuronal marker, Islet1 was used as our neurogenesis readout, because it is expressed in differentiating placode cells. Though I only tested Islet1, it is likely that DAPT causes changes in the expression of other neuronal genes in the opV placode.

In the chick retina, DAPT treatment caused a synchronized effect on the Notch pathway genes Hes1, Ngn2, and proneural genes like NeuroD. DAPT down-regulated Hes1 (which represses neurogenesis) and up-regulated the proneural gene Ngn2. Ngn2 then activated a cascade of proneural bHLH genes to initiate neurogenesis (Nelson et al. 2007). All of these genes (including Ngn2 and Hes1) are known to reside in the opV placode of chick embryos and are probably affected in a similar way by DAPT treatment. A similar model provides a plausible mechanism for neurogenesis in the opV placode. Cash1 and Ngn2 were singled out by Nelson, et al. as the genes necessary to start the proneural bHLH cascade in the chick retina (Nelson et al. 2007). Cash1 is not expected to be present in the opV placode, because Mash1 (Cash1's mammalian homologue) is known to reside in autonomic neural precursors and not sensory precursors (Ma et al. 1998). Conversely, Ngn2 is expressed in neuronal precursors of sensory neurons including the opV in chickens, so Ngn2 may be the key neuronal determination gene in the opV placode.

Another report seems to agree with this hypothesis, when Ngn1 (in chick Ngn2 is in the opV and Ngn1 is in the mmV, whereas in mice Ngn1 is in opV, and Ngn2 is in mmV) was knocked out in mice the trigeminal ganglion was absent due to the failure of opV cell delamination. It is known that the Notch pathway effects the expression of the Neurogenins and vise versa. Ngn1 was found to be upstream of Delta1 and the proneural bHLH genes NeuroD, NSCL1, SCG10. Because the expression pattern of Delta1 in opV is dependent on Ngn1 expression, it is likely that the Notch pathway is initially up-regulated by Ngn1. Interestingly, the initial expression pattern of Ngn1 shows a salt and pepper pattern of expression usually indicative of Notch signaling, but it was found that this expression did not rely on canonical Notch signaling.

Therefore, in chick it may be that Ngn2 is acting as a switch between progenitor cell and neuron in the opV placode. In future experiments it will be important to investigate the role of Notch signaling as it relates to Ngn2 in opV fate determination.

The finding that Pax3 cells were more abundant in the mesenchyme of DAPT treated heads was unexpected, but may be explained by a few hypotheses. First, DAPT could be causing Pax3 positive cells to proliferate and delaminate into the mesenchyme. To test whether this may be the case DAPT treated heads could be pulsed with BrdU before they were collected to see if the cells were cycling through S-phase in preparation for proliferation. To test for the possibility of cellular proliferation BrdU experiments are underway. A second and perhaps more likely hypothesis is that the cells in the surrounding ectoderm are being recruited to take the place of cells that have delaminated from the placode. That is, as cells differentiate and migrate to the mesenchyme, cells surrounding the placode up-regulate Pax3, thus compensating for a decrease in

ectodermal placode cells caused by delamination. These newly expressing Pax3 cells may then go on to express Islet1 and delaminate, allowing additional cells to compensate. This cycle would continue until many Pax3/Islet1 positive cells are observed. This hypothesis also agrees with previous data where removal of the opV placode results in healing over of the placode and rapid up-regulation Pax3 in ectoderm (Stark et al. 1997).

Before this report it was difficult to determine whether the neurons in the opV placode had the ability to express neuronal markers before they migrated from the placode, or whether migration was coupled to neurogenesis. At least one report states that neurogenesis is coupled to migration through Ngn2 in the mouse (Ge et al. 2006). Up-regulation of neuronal marker Islet1 in the ectoderm of DAPT treated embryos suggests that neuronal differentiation can take place before migration with certain treatments. Analysis of additional neuronal differentiation markers will help clarify whether the Islet1 positive cells we observed will continue to differentiate in the ectoderm.

Hes1 has been described in the opV placode at later stages of development than I tested (McCabe et al. 2004). I am currently testing the wild type expression pattern of Hes1 in the opV placode. Preliminary data suggests that Hes1 is present in the opV placode at the stages tested in this study. This suggests the possibility that Hes1 could be up-regulated by NICD in the opV placode, making this a possible mechanism for the neuronal inhibition phenotype observed. That is, NICD up-regulates Hes1, which inhibits neurogenesis.

Conclusions and Future Directions

I have shown that the Notch/Delta pathway plays a critical role in opV placode development. Without it the opV placode experiences increased differentiation, while over-activating the pathway causes cells to remain in the ectoderm and not differentiate. Therefore, the in wild type embryos the Notch pathway is likely acting to select the appropriate amount of cells to differentiate into neurons in the opV placode.

Using DAPT and NICD it will be interesting to test how the opV placode neuronal markers like Dll1, Ngn2, Neurofilaments, NeuroM, NeuroD and non-neuronal markers such as Hes1, Hes5 respond. This will give us a more complete picture of how the Notch/Delta pathway is acting in the ophthalmic trigeminal placode. Future experiments may also focus on how other pathways, such as Wnt and FGF, regulate the Notch pathway to control neurogenesis in the opV placode.

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Appendix

Table 3. NICD Cell Counts

9 Embryos	13 Placodes	Ectoderm					Mesenchyme				
Embryo	Section #	Pax3	All GFP	Pax3/GFP	GFP	Pax3/GFP/Islet1	Pax3	All GFP	Pax3/GFP	GFP	Pax3/GFP/Islet1
NICD 10-12ss 5-6-09 A	Top Placode	red	green+yellow	yellow	green only	red/green/blue	red	green+yellow	yellow	green only	red/green/blue
1	15	12	10	1	9	0	17	0	0	0	0
2	16	19	19	1	18	0	14	0	0	0	0
3	20	22	16	5	11	0	15	0	0	0	0
4	22	9	21	2	19	0	7	0	0	0	0
5	24	16	21	4	17	0	11	0	0	0	0
Total Cells		**78**	**87**	**13**	**74**	**0**	**64**	**0**	**0**	**0**	**0**
NICD 10-12ss 5-18-09 A	Left Placode										
1	15	11	18	6	12	0	8	1	1	0	1
2	17	19	39	16	23	0	5	0	0	0	0
3	18	13	41	14	27	0	5	0	0	0	0
4	20	5	19	2	17	0	6	1	0	1	0
5	21	4	10	1	9	0	14	1	0	1	0
Total Cells		**52**	**127**	**39**	**88**	**0**	**38**	**3**	**1**	**2**	**1**
NICD 10-12ss 5-18-09 E	Top/Left Placode										
1	19	20	12	3	9	2	19	5	2	3	0
2	24	11	15	1	14	0	16	0	0	0	0
3	27	13	3	1	2	1	13	0	0	0	0
4	28	9	11	1	10	1	30	8	3	5	0
5	29	17	18	4	14	4	16	1	0	1	0
Total Cells		**70**	**59**	**10**	**49**	**8**	**94**	**14**	**5**	**9**	**0**
NICD 10-12ss 5-18-09 E	Bottom Placode										
1	16	13	16	2	14	0	8	2	1	1	0
2	17	17	16	2	14	1	15	6	1	5	0
3	20	18	22	5	17	2	33	4	0	4	0
4	24	15	29	3	26	0	35	5	2	3	0
5	26	26	23	3	20	1	33	3	0	3	0
Total Cells		**89**	**106**	**15**	**91**	**4**	**124**	**20**	**4**	**16**	**0**
NICD 10-12ss 5-18-09 F	Right Placode										
1	3	4	24	1	23	0	10	3	0	3	0
2	4	3	24	1	23	0	11	4	0	4	0
3	5	10	29	2	27	2	11	2	0	2	0
4	6	4	22	1	21	1	11	2	0	2	0
5	9	7	25	2	23	1	10	3	1	2	0
Total Cells		**28**	**124**	**7**	**117**	**4**	**53**	**14**	**1**	**13**	**0**

NICD 10-12ss 5-18-09 G	Left Placode										
1	9	2	34	1	33	1	29	7	1	6	0
2	10	2	44	1	43	0	47	4	0	4	0
3	11	5	28	3	25	3	23	6	0	6	0
4	12	9	23	2	21	0	26	9	0	9	0
5	13	4	10	0	10	0	27	4	2	2	0
Total Cells		**22**	**139**	**7**	**132**	**4**	**152**	**30**	**3**	**27**	**0**
NICD 10-12ss 5-18-09 G	Right Placode										
1	19	8	15	2	13	2	17	7	0	7	0
2	20	5	17	2	15	1	31	5	2	3	0
3	22	6	23	2	21	2	28	3	1	2	1
4	23	7	33	4	29	3	47	3	1	2	0
5	24	6	29	6	23	1	43	1	0	1	0
Total Cells		**32**	**117**	**16**	**101**	**9**	**166**	**19**	**4**	**15**	**1**
NICD 10-12ss 5-18-09 H	Left Placode										
1	12	17	34	10	24	2	3	0	0	0	0
2	13	15	27	5	22	1	11	1	0	1	0
3	16	6	29	7	22	2	9	1	0	1	0
4	20	6	21	5	16	1	8	0	0	0	0
5	21	7	11	3	8	1	13	0	0	0	0
Total Cells		**51**	**122**	**30**	**92**	**7**	**44**	**2**	**0**	**2**	**0**
NICD 10-12ss 5-18-09 H	Right Placode										
1	13	9	17	4	13	3	7	0	0	0	0
2	16	5	27	5	22	1	9	0	0	0	0
3	21	16	6	3	3	1	11	0	0	0	0
4	23	11	4	1	3	1	2	0	0	0	0
5	24	10	9	0	9	0	6	0	0	0	0
Total Cells		**51**	**63**	**13**	**50**	**6**	**35**	**0**	**0**	**0**	**0**
NICD 10-12ss 5-18-09 J	Top Placode										
1	5	13	13	3	10	0	18	0	0	0	0
2	6	7	14	1	13	0	23	2	0	2	0
3	7	15	17	1	16	1	25	2	0	2	0
4	8	6	11	1	10	1	30	4	1	3	1
5	10	3	13	0	13	0	30	1	0	1	0
Total Cells		**44**	**68**	**6**	**62**	**2**	**126**	**9**	**1**	**8**	**1**

NICD 10-12ss 5-18-09 J	Bottom Placode										
1	2	6	9	0	9	0	11	0	0	0	0
2	4	6	24	1	23	0	11	4	2	2	0
3	5	7	41	0	41	0	13	3	0	3	0
4	6	9	35	4	31	0	6	2	0	2	0
5	7	3	33	2	31	0	9	2	1	1	1
Total Cells		**31**	**142**	**7**	**135**	**0**	**50**	**11**	**3**	**8**	**1**
NICD 10-12ss 5-20-09 B	Bottom Placode										
1	9	13	35	5	30	0	12	0	0	0	0
2	10	13	22	4	18	0	19	9	0	9	0
3	14	4	6	0	6	0	9	4	2	2	0
4	16	6	21	3	18	0	22	2	0	2	0
5	17	5	57	4	53	0	49	5	4	1	3
Total Cells		**41**	**141**	**16**	**125**	**0**	**111**	**20**	**6**	**14**	**3**
NICD 10-12ss 5-20-09 C	Top/Left Placode										
1	1	11	2	1	1	0	26	0	0	0	0
2	2	11	15	2	13	0	26	0	0	0	0
3	5	5	7	0	7	0	46	9	0	9	0
4	6	4	3	0	3	0	80	7	1	6	1
5	7	3	38	2	36	0	116	2	0	2	0
Total Cells		**34**	**65**	**5**	**60**	**0**	**294**	**18**	**1**	**17**	**1**

	Ectoderm					Mesenchyme				
	Pax3	All GFP	Pax3/GFP	GFP only	Pax3/GFP/Islet1	Pax3	All GFP	Pax3/GFP	GFP	Pax3/GFP/Islet1
Average of Total Cells	47.92	104.62	14.15	90.46	3.38	103.92	12.31	2.23	10.08	0.62
Standard Deviation	20.37	31.98	10.00	30.40	3.33	72.52	9.23	2.05	7.96	0.87

Table 4. PCIG Cell Counts

6 Embryos	10 Placodes	Ectoderm					Mesenchyme				
Embryo	Section #	Pax3	All GFP	Pax3/GFP	GFP only	Pax3/GFP/Islet1	Pax3	All GFP	Pax3/GFP	GFP only	Pax3/GFP/Islet1
		red	green+yellow	yellow	green only	red/green/blue	red	green+yellow	yellow	green only	red/green/blue
PCIG 10-12s 5-5-09 D	Top Placode										
1	16	12	4	3	1	0	52	20	17	3	16
2	20	0	8	0	8	0	38	9	5	4	5
3	21	0	9	0	9	0	32	17	14	3	14
4	22	1	7	0	7	0	28	18	16	2	15
5	23	0	6	0	6	0	21	11	10	1	10
Total Cells		**13**	**34**	**3**	**31**	**0**	**171**	**75**	**62**	**13**	**60**
PCIG 10-12s 5-5-09 D	Bottom Placode										
1	5	12	10	8	2	0	22	16	12	4	11
2	6	12	11	6	5	1	15	7	6	1	6
3	16	4	11	2	9	0	41	26	18	8	16
4	22	2	15	2	13	0	16	9	7	2	7
5	23	1	9	1	8	0	23	10	5	5	4
Total Cells		**31**	**56**	**19**	**37**	**1**	**117**	**68**	**48**	**20**	**44**
PCIG 10-12s 5-6-09 A	Bottom Placode										
1	17	24	45	23	22	1	108	53	48	5	42
2	19	8	34	3	31	0	80	53	35	18	37
3	25	2	30	2	28	0	124	80	64	16	62
4	28	2	34	0	34	0	102	69	58	11	49
5	32	3	28	2	26	0	112	39	35	4	34
Total Cells		**39**	**171**	**30**	**141**	**1**	**526**	**294**	**240**	**54**	**224**
PCIG 10-12s 5-18-09 A	Top Placode										
1	14	27	25	22	3	0	59	36	33	3	27
2	15	31	41	25	16	0	71	31	26	5	21
3	17	31	30	20	10	2	69	41	38	3	35
4	19	19	26	12	14	2	53	27	25	2	26
5	20	16	40	15	25	4	35	20	17	3	16
Total Cells		**124**	**162**	**94**	**68**	**8**	**287**	**155**	**139**	**16**	**125**

PCIG 10-12s 5-18-09 A	Bottom Placode										
1	20	26	22	14	8	0	83	58	52	6	51
2	24	18	29	15	14	1	92	64	63	1	56
3	27	14	22	10	12	1	67	46	37	9	39
4	28	15	24	10	14	4	110	58	52	6	46
5	29	14	23	13	10	1	63	66	51	15	48
Total Cells		**87**	**120**	**62**	**58**	**7**	**415**	**292**	**255**	**37**	**240**
PCIG 10-12s 5-18-09 B	Top Placode										
1	12	18	19	13	6	0	76	47	40	7	31
2	13	18	33	10	23	3	77	41	36	5	33
3	14	20	31	12	19	4	74	29	28	1	21
4	15	12	33	9	24	4	61	29	22	7	20
5	17	5	33	5	28	1	60	35	25	10	24
Total Cells		**73**	**149**	**49**	**100**	**12**	**348**	**181**	**151**	**30**	**129**
PCIG 10-12s 5-18-09 C	Top Placode										
1	15	41	73	40	33	4	52	48	36	12	34
2	16	37	44	22	22	0	66	40	32	8	24
3	19	11	20	7	13	0	47	34	27	7	21
4	20	15	40	10	30	0	67	43	37	6	30
5	22	21	36	18	18	2	40	27	21	6	20
Total Cells		**125**	**213**	**97**	**116**	**6**	**272**	**192**	**153**	**39**	**129**
PCIG 10-12s 5-18-09 C	Bottom Placode										
1	31	5	6	2	4	0	25	12	9	3	9
2	34	8	5	3	2	0	24	11	9	2	8
3	35	4	5	1	4	0	28	10	9	1	7
4	37	10	7	5	2	0	35	21	15	6	13
5	39	9	8	4	4	1	38	22	16	6	15
Total Cells		**36**	**31**	**15**	**16**	**1**	**150**	**76**	**58**	**18**	**52**

PCIG 10-12s 5-18-09 D	Top Placode										
1	14	30	46	22	24	4	61	42	32	10	32
2	15	28	32	18	14	2	64	39	30	9	28
3	16	17	29	10	19	1	55	38	32	6	17
4	20	20	38	9	29	4	33	28	21	7	19
5	23	19	45	8	37	2	35	14	8	6	8
Total Cells		**114**	**190**	**67**	**123**	**13**	**248**	**161**	**123**	**38**	**104**
PCIG 10-12s 5-18-09 D	Bottom Placode										
1	34	19	5	4	1	1	39	19	18	1	15
2	36	16	7	7	0	1	35	9	9	0	9
3	38	14	10	9	1	4	67	20	19	1	18
4	42	10	2	1	1	0	72	31	24	7	18
5	43	16	9	1	8	0	69	30	24	6	18
Total Cells		**75**	**33**	**22**	**11**	**6**	**282**	**109**	**94**	**15**	**78**
		Ectoderm					Mesenchyme				
		Pax3	All GFP	Pax3/GFP	GFP only	Pax3/GFP/Islet1	Pax3	All GFP	Pax3/GFP	GFP only	Pax3/GFP/Islet1
Average of Total Cells		71.70	115.90	45.80	70.10	5.50	281.60	160.30	132.30	28.00	118.50
Standard Deviation		40.90	71.16	33.30	47.17	4.70	124.65	83.14	71.96	13.68	67.82

Table 5. DAPT Cell Counts

10 Embryos	17 Placodes	Ectoderm			Mesenchyme		
Embryo	Section #	Pax3	Islet1	Pax3/Islet1	Pax3	Islet1	Pax3/Islet1
	Bottom Placode	red	green	red/green	red	green	red/green
DAPT 13-15s 1-17-09 D							
1	16	6	6	6	10	12	10
2	17	7	10	7	10	23	9
3	20	19	19	19	25	30	23
4	21	17	18	17	17	18	13
5	22	12	13	11	18	15	13
Total Cells		**61**	**66**	**60**	**80**	**98**	**68**
DAPT 13-15s 3-3-09 A	Left Placode						
1	18	15	19	13	7	15	7
2	19	18	24	18	17	23	17
3	20	17	18	16	8	12	8
4	21	12	16	12	26	28	26
5	23	15	18	15	18	29	17
Total Cells		**77**	**95**	**74**	**76**	**107**	**75**
DAPT 13-15s 3-3-09 A	Right Placode						
1	13	13	18	13	15	20	15
2	14	17	21	16	12	20	11
3	16	21	25	19	12	19	12
4	17	10	14	10	11	25	10
5	18	25	26	25	7	16	7
Total Cells		**86**	**104**	**83**	**57**	**100**	**55**
DAPT 13-15s 3-3-09 C	Top Placode						
1	21	29	24	24	44	53	44
2	23	27	30	26	39	43	39
3	24	27	36	27	61	65	54
4	25	20	24	18	66	80	65
5	27	20	33	20	75	90	75
Total Cells		**123**	**147**	**115**	**285**	**331**	**277**
DAPT 13-15s 3-3-09 C	Bottom Placode						
1	7	7	7	5	9	9	8
2	8	12	11	10	13	13	11
3	17	40	36	34	56	67	51
4	19	22	32	21	36	51	36
5	20	25	30	21	56	75	49
Total Cells		**106**	**116**	**91**	**170**	**215**	**155**

DAPT 13-15s 3-10-09 A	Top Placode						
1	11	25	27	22	71	72	68
2	12	34	42	34	78	95	75
3	13	16	24	16	73	69	67
4	14	31	39	30	68	93	61
5	15	26	31	24	57	100	56
Total Cells		**132**	**163**	**126**	**347**	**429**	**327**
DAPT 13-15s 3-10-09 B	Bottom Placode						
1	6	43	46	36	58	71	26
2	8	27	34	27	47	84	47
3	10	23	32	23	31	52	25
4	11	26	23	23	21	53	17
5	15	33	36	32	19	52	18
Total Cells		**152**	**171**	**141**	**176**	**312**	**133**
DAPT 13-15s 3-10-09 B	Top Placode						
1	15	40	43	40	25	38	22
2	17	42	50	42	29	71	28
3	19	36	42	34	22	57	22
4	21	34	31	28	47	62	38
5	22	37	43	36	34	56	31
Total Cells		**189**	**209**	**180**	**157**	**284**	**141**
DAPT 13-15s 3-10-09 C	Top Placode						
1	9	30	32	24	59	60	45
2	11	28	29	25	57	54	40
3	13	25	31	20	64	72	57
4	14	19	38	16	50	75	41
5	15	22	31	22	30	62	26
Total Cells		**124**	**161**	**107**	**260**	**323**	**209**
DAPT 13-15s 4-14-09 A	Bottom Placode						
1	3	12	11	10	15	25	13
2	4	17	19	15	35	47	34
3	5	20	21	19	34	40	33
4	6	19	21	18	48	57	43
5	9	21	20	19	52	75	50
Total Cells		**89**	**92**	**81**	**184**	**244**	**173**

DAPT 13-15s 4-14-09 A	Top Placode						
1	3	11	10	9	23	25	15
2	4	17	15	13	21	31	21
3	5	14	14	12	36	37	32
4	6	15	17	13	34	50	32
5	7	7	13	6	30	56	27
Total Cells		**64**	**69**	**53**	**144**	**199**	**127**
DAPT 13-15s 4-14-09 B	Left Placode						
1	3	11	13	11	24	35	23
2	5	14	21	14	23	66	20
3	8	19	21	18	38	75	37
4	10	14	23	14	39	56	37
5	12	12	18	17	21	69	10
Total Cells		**70**	**96**	**74**	**145**	**301**	**127**
DAPT 13-15s 4-14-09 B	Right Placode						
1	25	16	14	13	35	39	31
2	26	27	24	24	38	40	35
3	28	23	26	23	79	78	75
4	31	31	35	30	87	115	84
5	33	27	33	24	66	97	62
Total Cells		**124**	**132**	**114**	**305**	**369**	**287**
DAPT13-15ss 5-20-09 A	Bottom Placode						
1	10	42	36	34	34	35	33
2	13	55	74	53	33	62	32
3	15	72	64	61	67	80	66
4	16	40	50	38	50	67	49
5	19	20	36	19	42	62	41
Total Cells		**229**	**260**	**205**	**226**	**306**	**221**
DAPT13-15ss 5-20-09 A	Top Placode						
1	8	37	34	34	33	29	26
2	9	36	35	34	21	21	20
3	10	42	40	37	43	40	35
4	16	30	48	30	30	48	30
5	17	49	66	47	23	44	23
Total Cells		**194**	**223**	**182**	**150**	**182**	**134**

DAPT13-15ss 5-20-09 B	Top Placode						
1	8	28	38	28	60	66	60
2	9	52	51	50	30	47	30
3	10	48	57	48	36	46	34
4	12	43	49	40	67	67	60
5	17	42	49	37	28	67	27
Total Cells		**213**	**244**	**203**	**221**	**293**	**211**
DAPT13-15ss 5-20-09 B	Bottom Placode						
1	8	14	49	13	44	26	43
2	9	34	37	33	31	34	27
3	10	38	40	34	37	43	35
4	11	41	37	37	43	38	30
5	17	37	34	27	60	82	54
Total Cells		**164**	**197**	**144**	**215**	**223**	**189**
		Ectoderm			Mesenchyme		
		Pax3	Islet1	Pax3/Islet1	Pax3	Islet1	Pax3/Islet1
Average of Total Cells		129.24	149.71	119.59	188.12	253.88	171.12
Standard Deviation		53.47	60.83	49.30	80.96	95.42	77.47

Table 6. DMSO Cell Counts

9 Embryos	17 Placodes	Ectoderm			Mesenchyme		
Embryo	Section #	Pax3	Islet1	Pax3/Islet1	Pax3	Islet1	Pax3/Islet1
DMSO 13-15s 1-17-09 E	Top/Left Placode	red	green	red/green	red	green	red/green
1	14	17	0	0	5	7	5
2	22	23	0	0	8	8	8
3	26	10	2	0	10	10	9
4	27	14	1	1	13	12	12
5	28	21	0	0	17	19	13
Total Cells		**85**	**3**	**1**	**53**	**56**	**47**
DMSO 13-15s 1-17-09 E	Bottom/Right Placode						
1	7	18	5	4	6	7	6
2	12	8	3	2	7	8	7
3	19	11	1	1	10	11	10
4	20	9	1	1	13	11	10
5	21	32	1	1	11	13	11
Total Cells		**78**	**11**	**9**	**47**	**50**	**44**
DMSO 13-15s 3-10-09 A	Top/Left Placode						
1	9	14	3	3	6	6	6
2	11	23	7	7	16	11	11
3	12	19	2	2	17	11	11
4	14	29	2	2	14	14	14
5	19	6	1	1	13	12	11
Total Cells		**91**	**15**	**15**	**66**	**54**	**53**
DMSO 13-15s 3-10-09 A	Bottom/Right Placode						
1	6	17	4	4	3	3	3
2	7	15	4	4	2	2	2
3	8	20	5	5	8	8	8
4	9	23	4	4	9	7	7
5	15	21	1	1	19	15	14
Total Cells		**96**	**18**	**18**	**41**	**35**	**34**
DMSO13-15s 2-3-09	Top/Left Placode						
1	12	23	4	4	9	14	9
2	14	29	0	0	11	18	11
3	20	27	6	3	10	10	10
4	22	27	0	0	7	8	8
5	28	50	3	3	12	10	10
Total Cells		**156**	**13**	**10**	**49**	**60**	**48**

DMSO13-15s 2-3-09	Bottom/Right Placode						
1	11	31	1	1	14	11	11
2	12	48	6	4	14	20	16
3	14	40	1	1	17	19	12
4	23	29	7	7	22	11	11
5	27	23	4	2	12	12	8
Total Cells		**171**	**19**	**15**	**79**	**73**	**58**
DMSO13-15s 3-3-09 A	Top/Left Placode						
1	1	13	1	1	11	12	11
2	3	8	0	0	9	12	8
3	6	7	1	1	5	9	5
4	8	15	0	0	8	8	7
5	9	11	0	0	8	10	8
Total Cells		**54**	**2**	**2**	**41**	**51**	**39**
DMSO13-15s 3-3-09 A	Bottom/Right Placode						
1	1	11	2	1	8	11	8
2	3	11	4	4	3	5	3
3	5	10	0	0	10	11	10
4	9	10	0	0	11	15	10
5	10	8	0	0	8	11	8
Total Cells		**50**	**6**	**5**	**40**	**53**	**39**
DMSO13-15s 4-14-09 B	Bottom Placode						
1	12	39	4	3	26	26	23
2	13	36	4	1	28	32	25
3	14	44	2	2	28	28	22
4	15	43	1	1	15	22	14
5	19	30	2	2	16	20	16
Total Cells		**192**	**13**	**9**	**113**	**128**	**100**
DMSO13-15s 4-14-09 B	Top/Right Placode						
1	44	38	1	1	16	26	16
2	46	52	2	2	28	30	28
3	47	27	2	1	17	31	16
4	48	32	2	2	22	24	20
5	49	24	3	2	20	24	19
Total Cells		**173**	**10**	**8**	**103**	**135**	**99**

DMSO13-15s 5-5-09 A	Bottom Placode						
1	11	14	5	4	11	9	7
2	12	17	4	2	14	15	13
3	17	28	2	2	9	9	7
4	18	37	4	3	20	19	14
5	20	41	2	1	18	14	7
Total Cells		**137**	**17**	**12**	**72**	**66**	**48**
DMSO13-15s 5-5-09 A	Top Placode						
1	24	22	2	1	2	2	1
2	26	20	1	1	2	2	2
3	27	20	1	1	6	7	6
4	28	15	2	1	19	11	11
5	30	19	1	0	9	13	7
Total Cells		**96**	**7**	**4**	**38**	**35**	**27**
DMSO13-15s 5-5-09 C	Bottom/Right Placode						
1	35	24	0	0	18	20	16
2	37	31	1	1	25	26	24
3	39	23	0	0	14	16	14
4	40	17	0	0	17	17	17
5	42	20	3	3	14	22	14
Total Cells		**115**	**4**	**4**	**88**	**101**	**85**
DMSO13-15s 5-5-09 C	Top/Left Placode						
1	9	24	2	2	16	15	13
2	10	37	4	2	18	21	16
3	11	32	3	3	14	18	14
4	12	44	1	1	12	16	12
5	28	22	5	4	29	29	27
Total Cells		**159**	**15**	**12**	**89**	**99**	**82**
DMSO13-15s 5-5-09 D	Top/Left Placode						
1	13	31	0	0	22	18	14
2	17	41	3	3	25	28	23
3	19	36	5	5	19	22	17
4	21	34	5	4	30	35	28
5	25	29	2	2	27	30	27
Total Cells		**171**	**15**	**14**	**123**	**133**	**109**

DMSO13-15s 5-5-09 D	Bottom/Right Placode						
1	13	25	3	3	28	25	18
2	17	20	0	0	24	21	20
3	22	18	1	1	14	11	11
4	25	31	2	2	24	26	21
5	27	32	3	3	27	30	24
Total Cells		**126**	**9**	**9**	**117**	**113**	**94**
DMSO13-15s 5-5-09 F	Top Placode						
1	2	26	3	3	18	18	17
2	4	18	0	0	38	31	26
3	6	27	0	0	16	14	11
4	7	18	2	2	32	30	23
5	8	21	3	2	27	26	21
Total Cells		**110**	**8**	**7**	**131**	**119**	**98**
		Ectoderm			Mesenchyme		
		Pax3	Islet1	Pax3/Islet1	Pax3	Islet1	Pax3/Islet1
Average of Total Cells		121.18	10.88	9.06	75.88	80.06	64.94
Standard Deviation		43.79	5.33	4.88	32.35	35.33	27.65

Curriculum Vitae of
Matthew K. Ball

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Education

I have been accepted to attend medical school at the Arizona College of Osteopathic Medicine from August 2009 – May 2013.

Master's degree in Physiology and Developmental Biology from Brigham Young University
August 2007 – August 2009
GPA: 3.89

Bachelor's degree in Biology from Brigham Young University – Idaho in Biology.
Minor is Chemistry
August 2000 – August 2006 (from March 2001 – March 2003 I served a religious mission)
GPA: 3.53

Laboratory/Work Experience

Teaching and Research Assistantships at BYU– I worked in the lab of Dr. Michael Stark doing research on the development of the ophthalmic trigeminal placode, which forms part of the trigeminal cranial nerve. My research focused on the role of the Notch pathway in forming sensory neurons. I was also a teacher's aid for Dr. Stark's Developmental Biology, PDBio 482 class for two semesters.

Reference: Dr. Michael Stark Ph.D. (801) 422-7498
Department of Physilogy and Developmental Biology at Brigham Young University
Provo, UT 84602
Worked: Part-Time and Full-Time, August 2007 – August 2009
Position: Teacher's Assistant and Research Assistant
Skills, Responsibilities, and Training:

- Microsurgeries on chick embryos
- Tissue Culture (Orgaotypic Explants)
- In situ analysis of RNA expression patterns in embryos
- Immunohistochemial staining and analysis of protein expression patterns
- Frozen Sectioning
- DNA extraction
- DNA cloning
- In ovo DNA electroporation of chick embryos
- Florescent microscopy

From Sept 2006 to July 2007 I worked as a residential framer and mechanic to support my wife through school.

Medical Research Internship at Idaho State University – I studied the developmentally defective effects of thalidomide on developing limb buds of chick embryos. I also did microsurgeries on the developing beaks of chicks to implant beads soaked with growth factors. We were attempting to induce the beak to grow teeth like structures. This internship was awarded

through a National Institutes of Health grant to the IDeA Network of Biomedical Research Excellence (INBRE) program.

Reference: Dr. Trent Stephens Ph.D. (208) 282-3993
Department of Biological Sciences at Idaho State University
Pocatello, ID 83209

Worked: Full-Time, May 2006 – August 2006

Position: Intern

Skills, Responsibilities, and Training:

- Microsurgeries on chick embryos
- Bead implantation

I have worked for many other employers before the dates mentioned here. A full list of employment, skills and contacts is available upon request.

Awards

- 2006 ($3000) Student Research Award from the National Institutes of Health grant to the IDeA Network of Biomedical Research Excellence (INBRE)

- 2009 ($100) Student Travel Award granted by the Society for Developmental Biology

Presentations

Title: *Function of the Notch/Delta Pathway in Ophthalmic Trigeminal Placode Development*
Presented at the Physiology and Developmental Biology Seminar of Brigham Young University, January 29, 2009.

Abstracts

Title: *Function of the Notch/Delta Pathway in Ophthalmic Trigeminal Placode Development*
Presented at the Society for Developmental Biology 68th Annual Meeting, held at the Hyatt Regency, San Francisco, CA July 23-27, 2009.

Society Membership

2008 and 2009 Member of the Society for Developmental Biology

Extracurricular and Leadership Activities

School Involvement and Leadership:

Fall 2005 Pre-Medical Society Member, Men's Choir Member
Winter 2005 Serving with Smiles Committee Member, Student Alumni Networking Committee Member, Center Stage Committee Member, Men's Choir Member
Fall 2004 Student Alumni Networking Committee Coordinator, Center Stage Committee Member, Men's Choir Member, Cross-Country
Summer 2004 Student Alumni Student Legacy Endowment Committee Member, Men's Choir Member
Winter 2004 Men's Choir Member
Fall 2003 Men's Choir Member, Cross-Country

Description of Organizations and Volunteer Experiences:

Men's Choir Member – The Brigham Young University - Idaho Men's Choir sings songs that entertain and inspire. While performing for our school and the community, I have learned the value of working in unison towards a common goal.

Student Alumni, Legacy Endowment Committee Member – The Student Alumni Association fosters school spirit, loyalty, and service. The committee I was on raised funds for students in financial need. This organization taught me the value of working in a council to come up with the best ideas to meet our organization's goals.

Student Alumni, Networking Committee Coordinator – In this committee we sought for ways to link our school's alumni to the students. This position was difficult, because the committee did not exist before I became the coordinator. I learned how to plan and work under the burden of increased responsibility.
Center Stage Committee Member – This committee coordinated the public relations for all entertainment coming from off campus BYU-Idaho. We were the community and student's connection with those events. Our committee discussed promotional ideas and put the ideas to practice.
Serving with Smiles Committee Member – This committee planned and carried out regular service projects benefiting BYU-Idaho and the surrounding community. I learned how to plan and implement projects on a large scale.
Student Alumni Networking Committee Member – I was originally the committee coordinator for this committee, but the coordinators change on a semester by semester basis, so I became a committee member. I learned how to inspire new leaders and help them implement their ideas.

Church Involvement and Leadership

- Councilor in a presidency serving a group of about 40 male members
- Secretary to two bishops
- Teacher of various religious classes