Neuropeptides: Developmental Signals in Placode Progenitor Formation

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http://dx.doi.org/10.1016/j.devcel.2013.07.001

SUMMARY

Few families of signaling factors have been implicated in the control of development. Here, we identify the neuropeptides nociceptin and somatostatin, a neurotransmitter and neuroendocrine hormone, as a class of developmental signals in both chick and zebrafish. We show that signals from the anterior mesendoderm are required for the formation of anterior placode progenitors, with one of the signals being somatostatin. Somatostatin controls ectodermal expression of nociceptin, and both peptides regulate Pax6 in lens and olfactory progenitors. Consequently, loss of somatostatin and nociceptin signaling leads to severe reduction of lens formation. Our findings not only uncover these neuropeptides as developmental signals but also identify a long-sought-after mechanism that initiates Pax6 in placode progenitors and may explain the ancient evolutionary origin of neuropeptides, predating a complex nervous system.

RESULTS

Identification of Somatostatin and Nociceptin as Potential Developmental Signals

Consistent with its function, Pax6 is initially confined to future lens and olfactory cells (aPPs). However, chick posterior placode progenitors (pPPs; inner ear and epibranchial ganglia) upregulate Pax6 within only 5 hr when cultured in isolation and ultimately turn into lenses (Bailey et al., 2006), providing an experimental paradigm to screen for new Pax6 regulators. Transcriptome comparison of four different cell populations (HH6 aPPs and pPPs before and after 5 hr culture) reveals 136 Pax6 coregulated transcripts. Among these, only four encode signaling molecules, including the propeptide for the opioid-related Noc (Figure S1 available online); the receptor for another neuropeptide, SST, is enriched in aPPs. This raises the intriguing possibility that in addition to their well-known functions in the adult nervous system and neuroendocrine modulation (Gahete et al., 2010), they may also play a role during development.

We therefore surveyed the expression of Noc and SST and their receptors from primitive streak to early somite stages. The SST prepropeptide and the processed peptide are expressed in the anterior mesendoderm underlying Pax6+ aPPs (Figures 1A–1C, 1A’, S1Ca–S1Cf, S2A, and S2B). In contrast, its receptor SSTR5 and Noc (Figures 1D–1I, 1F–1L) are restricted to aPPs in the overlying ectoderm, where they colocalize with Pax6+ (Figures 1J–1L); both are downregulated rapidly in aPPs after the HH8. Thus, Noc and SSTR5 represent aPP-specific transcripts. Like Noc mRNA, the processed
peptide is present in the ectoderm (Figures S1Cg–S1Cl). Noc signaling is mediated by its cognate G protein-coupled receptor opiate receptor-like 1 (OPRL1) and, due to a change in the N-terminal amino acid in most nonmammalian vertebrates, by δ, κ, and μ opioid receptors (Danielson et al., 2001) (Figures S1Aa and S1Ab). At neural plate stages, all opioid and other SST (STTR1, STTR3, STTR4) receptors are broadly expressed in the ectoderm including placode progenitors (Figures S2Ac–S2Aj and S3Aa–S3Al). Thus, the expression of both neuropeptides and their receptors is consistent with a role in initiating Pax6 expression and in specifying progenitors for the olfactory epithelium and the crystalline lens.

**Somatostatin Signaling from the Anterior Mesendoderm Promotes Placode Progenitor Fate**

SST is expressed in the anterior mesendoderm, a tissue implicated in forebrain patterning (Dale et al., 1997; Foley et al., 1997; Wilson and Houart, 2004; Withington et al., 2001). To test whether mesendoderm-derived signals are required for aPP identity, we ablated this tissue unilaterally in HH4+/HH5/C0 embryos (Figures 2A, S2Ba, and S2Bb). We find that Pax6 (1 out of 14, 7% Pax6+; Figures 2B and 2B0) and the general PP marker Eya2 (0 out of 4 Eya2+; Figure S2Be) are absent 5–6 hr thereafter, whereas sham-operated embryos are normal (Figure S2Bf). Likewise, Noc transcripts are reduced at 5–6 hr and completely lost 16 hr after ablation (0 out of 7 Noc+; Figures 2C and 2C0) as is SSTR5 (0 out of 9 SSTR+; Figures 2D and 2D0). In contrast, the posterior ectoderm marker Gbx2 (n = 4) and nonneural ectoderm marker Dlx5 (n = 4) are unaffected (Figures S2C and S2D). Can SST rescue the expression of aPP markers? After mesendoderm removal, local exposure to SST-, but not DMSO-, coated beads (Figures S2Bg–S2Bh) rescues Pax6 (3 out of 3 Pax6+; Figures 2E–2E0) and Noc expression (5 out of 5 Noc+; Figures 2F and 2F0) after 16 hr culture. Thus, the anterior mesendoderm provides key signals to promote aPP identity, SST being one of these signals.

To assess whether SST signaling is required for aPP character, we used two different approaches. First, HH4 embryos
were cultured with the SST antagonist cyclosomatostatin (CSST) or vehicle control (DMSO). At HH6/HH7, Pax6 expression is absent in CSST (4 out of 18, 22% Pax6+; Figure 2H) but present in DMSO-treated controls (6 out of 6 Pax6+; Figure 2G). Second, we asked whether the receptor SSTR5 mediates SST function. Control or SSTR5 translation-blocking morpholinos (MOs) were electroporated into future aPPs at HH4. Like mesendoderm ablation, SSTR5 knockdown leads to a loss of Pax6 (1 out of 13, 8% Pax6+; Figures 2I and 2J), Noc (5 out of 19, 26% Noc+; Figures 2K and 2L), and Eya2 (0 out of 6 Eya2+; Figures 2M, 2N, and 2N0) at HH6-8, whereas control MOs have no effect (Figures S2Bi–S2Bn). Together, these results show that SST signaling from the anterior mesendoderm is crucial for the specification of lens and olfactory progenitors by controlling the onset of Pax6 and other PP-specific transcripts.

**Signals from the Posterior Head Mesoderm Repress aPP Markers**

Although Pax6 and Noc are upregulated in explanted pPPs (Figures S1 and 3A), they are restricted to the aPP region in the embryo (Figure 1), suggesting that anterior fates are actively repressed in vivo. A possible source for such repressive signals is the mesoderm underlying pPPs. To test this, we analyzed Pax6 and Noc expression in pPP explants cultured with and without posterior mesoderm (pM). Indeed, we find that both transcripts are repressed by mesoderm-derived signals (Figure 3A). pPPs normally give rise to otic and epibranchial placodes with FGFs being potent inducers of a common otic-epibranchial progenitor domain (Freter et al., 2008; Groves and Bronner-Fraser, 2000; Martin and Groves, 2006; Yang et al., 2013). To test whether FGF signaling is sufficient for aPP inhibition, we cultured pPP explants with FGF2; this...

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*Figure 2. SST Is Required for aPP Character*

(A) Unilateral ablation of axial and paraxial mesendoderm (red line); light gray indicates mid- and hindgut endoderm, and gray shows foregut endoderm. (B–F) Pax6 (B and B'), Noc (C and C'), and SSTR5 (D and D') after mesendoderm ablation (dotted lines); Pax6 (E and E') and Noc (F and F') after mesoderm ablation and SST bead graft. SST-coated beads (*) restore Pax6 (E–E') and Noc (F and F') after ablation (arrowheads). Lines in (B)–(F) indicate the level of sections shown in (A')–(E').

(G and H) Pax6 after DMSO (G) or CSST (H) treatment.

(I–N) Pax6 (I and J), Noc (K and L), and Eya2 (M, N, and N') in SSTR5 morphants. (J), (L), and (N) show the same embryos as in (I), (K), and (M), respectively, after MO detection (brown).

See also Figure S2.
induces the otic-epibranchial marker Pax2 (data not shown) (Freter et al., 2008; Groves and Bronner-Fraser, 2000; Martin and Groves, 2006; Yang et al., 2013). Although Noc expression does not change significantly when compared to controls, Pax6 is significantly repressed (Figure 3A). To test whether mesoderm-derived FGF signaling is required for Pax6 repression, we treated pPP/pM explants with the FGF antagonist SU5402; this rescues Pax6 expression partially but does not restore the expression levels observed in the absence of mesoderm (Figure 3A). Together, these findings show that posterior head mesoderm plays a role in patterning the placodal domain by simultaneously promoting posterior and repressing anterior character. Although FGF signaling is involved in this process, other unidentified pathways must cooperate.

Figure 3. Noc Is Required for aPP Character
(A) Regulation of aPP markers in vitro. (a) Quantification of Noc and Pax6 expression by NanoString nCounter in pPP explants cultured alone (black), with FGF2 (red), with posterior head mesoderm (pM; gray), or with posterior head mesoderm and SU5402 (blue). Bars represent means of normalized values ± SE. The asterisk (*) indicates significant differences: p < 0.05. (b) Cultured pPP explants initiate Noc (c) and Pax6 after DMSO (c) or TRAP101 (d) treatment. (e–g) Pax6 in explants cultured with control MOs (e), Noc splice-blocking MOs (f), or Noc MOs and peptide (g). (h) Graph showing Noc knockdown effect on Pax6 and Six1. Bars represent mean values ± SE. The asterisk (*) indicates significant differences: p < 0.05. See also Figure S3.
(B) Noc is required for aPP fate in vivo. (a and b) HH4+/HH5/C0 embryos cultured for 12 hr in DMSO (a) or opioid receptor inhibitors (b; N/U: naloxone, UFP101), (c and d) Pax6 in HH4+/HH5 embryos treated with DMSO (c) or CSST (C) for 36 hr, naloxone (N) and UFP101 (U); compare brackets in (c) and (d). (e–h) z-crystallin expression in embryos cultured for 60 hr from HH4+/HH5 with DMSO (e) or CSST (C), naloxone, and UFP101 (f–h). (e–h) show the range of phenotypes, and (e’–h’) show sections through the left and right lens (L) regions of the same embryos shown in (e–h). OV, optic vesicle. (i–k) After electroporation of Noc ATG MOs at HH4, embryos were cultured for 10 hr (i and i’), and k and k’). Brown (i’ and k’) and green (j’) indicate electroporated cells.
Nociceptin Promotes aPPs

Noc was identified as a Pax6 coregulated gene (Figure S1); both genes are coexpressed in explanted pPPs (Figure 1), and like Pax6, Noc is rapidly upregulated in control pPP explants (70% of control explants). To test whether Pax6 upregulation depends on Noc signaling, we used an OPRL1 antagonist. Pax6 transcripts are present in DMSO-treated control pPP explants (17 out of 32; 53% Pax6⁺; Figure 3Ab) but absent when Noc signaling is inhibited (11 out of 15, 35% Pax6⁺; Figure 3Ad). Likewise, Noc splice MOs, but not control MOs, prevent Pax6 initiation (Figures 3 Ae, 3Af, and 3Ah; controls, 39 out of 64, 61% Pax6⁺; experimental, 27 out of 73, 34% Pax6⁺). This effect is rescued by the addition of Noc peptide (13 out of 17, 76% Pax6⁺; Figures 3Ag, 3Ah, and 3Sc), demonstrating the specificity of the MOs. In contrast, the generic PP marker Six1 does not change after Noc knockdown (Figure 3Ah, 23 out of 23 Six1⁺; Figure 3ScA), indicating that Noc specifically promotes anterior character.

To confirm that Noc is required for aPP specification in vivo, we used two strategies. First, HH4 chick embryos were treated with OPRL1 and opiate receptor antagonists; this leads to loss of Pax6 at HH6 (Figure 3Bb; 0 out of 8 Pax6⁺), unlike DMSO-treated controls (Figure 3Ba; 8 out of 11, 72% Pax6⁺). Second, we electroporated translation- or splice-blocking Noc MOs, alone or in combination, into stage HH4 chick embryos, targeting future lens and olfactory cells. These produce identical phenotypes: at early somite stages, the expression of general placode progenitor markers (Figures S3Ba, S3Bb, and S3Bb'; n = 5), the neural crest marker Pax7 (Figures S3Ba, S3Bb, and S3Bb; n = 5), and Otx2 (Figures S3Bc, S3Bd, and S3Bd; n = 7), an anterior ectoderm marker expressed prior to Pax6 and Noc, are unaffected. However, like in vitro, in Noc morphants, Pax6 is severely reduced in lens and olfactory progenitors at head process and early somite stages (Figures 3BI–3Bj; 7 out of 14, 50% Pax6⁺; Figure 3Scb) as is Noc itself (0 out of 4 Noc⁻; Figures 3Bb and 3Bj), whereas control MOs have little effect (Figures S2I–S2L; 7 out of 10, 70% Pax6⁺; 7 out of 11, 64% Noc⁻). In contrast, the CNS domain of Pax6 is unaffected as is Six3 (n = 9; data not shown) and Gaf (n = 2; data not shown) in the forebrain. Thus, Noc does not influence neural plate formation but is required to regulate anterior placode fates by controlling its own expression and that of the master regulator Pax6.

So far, our results show that mesendoderm-derived SST initiates Noc expression in the overlying ectoderm and that both signals are required for aPP-specific gene expression. Do SST and Noc act in a linear pathway? If so, nociceptin should rescue the SST phenotype. We therefore ablated anterior mesendoderm (the source of SST) or knocked down SSTR5 followed by a graft of Noc-coated beads. Activation of Noc signaling does not rescue Pax6 expression in the absence of SST (Figure S3D; n = 13). Thus, the two neuropeptides act in parallel, and both are required for Pax6 expression and, consequently, for aPP specification.

Lens Defects in the Absence of SST and Noc Signaling

SST and Noc are transiently expressed in the anterior mesendoderm and anterior preplacodal ectoderm, respectively, and participate in aPP specification by regulating Pax6 (see above). Does the loss of aPP character affect placode formation at later stages? To assess this, we treated embryos with inhibitors of Noc and SST signaling starting at HH4/HH5. This reduces Pax6 in the surface ectoderm, but not in the brain, at HH10/HH11 (Figures 3Be and 3Bd; 7 out of 14, 50% Pax6 reduction; controls: 13 out of 14, 93% normal). At embryonic day 3 (E3), phenotypes vary slightly, with only 12.4% (n = 24) showing normal expression of the lens differentiation marker b-crystallin compared to 70% of DMSO-treated controls (n = 20). Lens placodes or vesicles are absent uni- (12.4%) or bilaterally (16.6%) or are substantially smaller (58.3%; Figures 3Bf–3Bh and 3Bf–3Bh') than in controls (Figures 3Be and 3Bf; n = 20; 30% with unilateral small placode). Although patches of b-crystallin show typical placode morphology, they remain small and never invaginate to form a vesicle. In severe cases, optic vesicle morphology is affected because lens-derived signals are required for its normal development (Coulombre and Coulombre, 1964; Yamamoto and Jeffery, 2000; Chow and Lang, 2001). Thus, SST and Noc signaling are critical for aPP specification and therefore for normal lens formation.

Somatostatin and Nociceptin Functions Are Conserved in Zebrafish

To assess whether neuropeptide function in anterior placode precursor formation is conserved across species, we turned to zebrafish and cloned prepronociceptin b (pnocb). As in chick, pnocb is coexpressed with Pax6b in aPPs (Figures 4A and 4B), whereas SST1 (Devos et al., 2002) is expressed in the mesendoderm (Figure 4C). Like in chick, inhibition of SST signaling using the antagonist CSST leads to disruption of Pax6b at neural plate stages in a dose-dependent manner (Figures 4D, 4E, and 4O). Noc knockdown using different MOs alone or in combination results in uni- or bilateral reduction of Pax6b (Figures 4G, 4H, and 4O; 32 out of 171, 19% normal Pax6b and Pitx3 (Figure 4P; 5 out of 58, 9% Pitx3⁻) in aPPs at neural plate stages. Pax6b expression is rescued by growing morphants in the presence of Noc peptide (Figures 4I–4K and 4O). In pnocb morphants, the general PP markers Eya1 and Six1 are unaffected (data not shown), suggesting that, like in chick, aPPs retain placode progenitor identity but lose their anterior character. In contrast to Pax6b reduction in aPPs, its expression in the neural plate is unaffected, as are the neural markers Rx3, Emx1, Six3, and lnxb until at least the ten somite stage (data not shown; n > 25 for each marker). These results show that nociceptin signaling primarily affects placode, but not neural plate, development.

Consistent with the loss of early Pax6b, Noc morphants show variable eye phenotypes after 24 hr: their lenses are smaller, asymmetric, or absent (n = 84; Figures 4L–4N and 4P), and as a consequence, the optic vesicles are reduced in size. Simultaneous inhibition of SST and Noc phenocopies the loss of each pathway individually (n = 54; data not shown). Thus, like in chick, Noc is coexpressed with Pax6b in placode progenitors at the border of the anterior neural plate, whereas SST1 is expressed in the underlying mesendoderm. Both contribute to the specification of aPPs, and their loss leads to abnormal eye development in chick and zebrafish.

DISCUSSION

Our findings reveal a function for the anterior mesendoderm in controlling aPP fates as an early step for lens and olfactory development.
Figure 4. SST and Noc Control aPPs in Zebrafish 

(A–C) Expression of Pax6b (A), pnocb (B), and SST1 (C) in zebrafish at early somite stages; dorsal views, anterior to the left. 

(D and E) Pax6b reduction by CSST (E; arrowhead), but not by DMSO (D; white arrowhead). 

(F–H) Embryos were injected with control (F) or pnocb MOs (G and H); the latter show Pax6b reduction (arrowheads in G and H). Dotted lines indicate CNS expression of Pax6b. 

(I–N) pnocb ATG (I–K) or control (L–N) MO-injected embryos were incubated in DMSO (I and L) or Noc peptide (J, K, M, and N). Dotted lines indicate CNS Pax6b expression. 

(O–Q) Embryos were injected with control (O) or pnocb MOs (P and Q). At 24 hpf (frontal views), Pitx3 expression reveals asymmetric, small (P; arrowhead) or almost absent lenses (Q; arrowhead). 

(legend continued on next page)
development. We identify two neuropeptides, SST and Noc, mediating this process in amniotes and anamniotes (Figure 4T). Mesendoderm-derived SST promotes aPP identity in the overlying ectoderm by regulating Eya2 and Noc. In turn, Noc controls its own expression and, together with SST, the onset of Pax6, a key regulator of eye and olfactory fates.

Mice lacking Noc, SST, and their receptors have been generated (Köster et al., 1999; Low et al., 2001; Nishi et al., 1997; Zeyda et al., 2001; Zeyda and Hochgeschwender, 2008). Adult animals do not display obvious sense organ phenotypes, for which several other explanations are possible. First, the mutants have not been examined for defects in lens and olfactory progenitors—the effects of the mutants may be transient or subtle. With respect to SST, a robust phenotype would only be expected when all four SSTRs expressed in sensory progenitors are ablated. Furthermore, a second peptide, cortistatin, is often coexpressed with SST, signals through all SSTRs, and may thus compensate for the absence of SST (Gahete et al., 2008; Zeyda and Hochgeschwender, 2008). Finally, the mammalian Noc prepropeptide contains a second peptide, nocistatin, a Noc antagonist, which is absent in nonmammalian vertebrates (Danielson et al., 2001; Okuda-Ashitaka et al., 1998). This may account for subtle or lack of phenotype in mice. Here, using different tools including tissue ablation, morpholinos, and drugs to interfere with SST and Noc function, we reveal their role in aPP specification. Whether neuropeptides have a similar function in rodents and other mammals remains to be discovered; however, our results are consistent in both amniotes and anamniotes.

Once placode progenitors are specified, they have an autonomous tendency to form a lens when cultured in isolation, regardless of their later fate (Bailey et al., 2006). They do so by initiating Pax6 followed by the transcription factors controlling lensless of their later fate (Bailey et al., 2006). They do so by initiating results are consistent in both amniotes and anamniotes. Whether neuropeptides have a similar function in rodents and other mammals remains to be discovered; however, our results are consistent in both amniotes and anamniotes.

Neuropeptides in Olfactory and Lens Precursors

EXPERIMENTAL PROCEDURES

Chick Embryo Experiments

All chick experiments involve embryos younger than E10 and do not require a UK Home Office license. Embryos were cultured in New (Stern and Ireland, 1981) or Cornish (Nagai et al., 2011) paistry culture. Mesendoderm ablations were performed at HHS+. Beads coated with SST, nociceptin, or DMSO (control) were grafted into the ablated area.

CSST (cyclo(7-aminohexanoyl-Phe-D-Trp-Lys-Thr[Bzl]); Sigma-Aldrich) and naloxone (Sigma-Aldrich) were prepared in DMSO (1 mM), UFP101 (Sigma) in H2O (1 mM). For inhibition studies, embryos were preincubated for 1 hr in SST and/or Noc antagonists (1 μM each) or DMSO (0.1%; control) and cultured in their presence for 5–60 hr. For knockdown, experiments were performed using MOs (Gene Tools), which were electroporated as described by Voiculescu et al. (2008).

HHS+-6 pPP explants were cultured as described by Bailey et al. (2006). Collagen gels and culture media were supplemented as required with nociceptin antagonists, nociceptin, MOs, FGF2, or SU5402. For coculture with posterior head mesoderm, pPP and mesoderm were dissected separately and recombined before culture.

Embryos and explants were processed for in situ hybridization (Streit et al., 1998) using DIG-labeled antisense probes. For histological sections, embryos were embedded in paraffin and sectioned transversally at 15 μm.

NanoString nCounter

For each experimental condition, eight to ten pPP explants were lysed in lysis buffer (Ambion). Total RNA was hybridized with capture and reporter probes according to the nCounter Gene Expression Assay Manual. Following washing, target/probe complexes were immobilized for data collection in the nCounter Digital Analyzer. Each experiment was repeated three times on
independent occasions. Mean value ± SD for Pax6 and Noc was extracted from the normalized data.

**Cloning of Zebrafish pnocb and Functional Experiments**

All zebrafish studies were performed with approval from the UK Home Office under a HO project license to C.H. Zebrafish pnocb was cloned by RT-PCR from 48 hpf embryo cDNA. pnoc or control MOs were injected at the one- to four-cell stage at a concentration of 1.8 ng/embryo. Embryos were grown at 28°C until the desired stage. For inhibition and rescue experiments, dechorio-nated embryos were incubated in appropriate compounds from 4 to 5 hpf until they had reached the desired stage.

**ACCESSION NUMBERS**

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE48116.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.07.001.

**ACKNOWLEDGMENTS**

The authors thank Ewa Kolano-Merlin and Vicky Snowden for excellent technical support, Timothy Grocott and Tatjana Sauka-Spengler for help with NanoString nCounter, and Claudio Stern, Ben Steventon, and Timothy Grocott for critical reading of the manuscript. This work was supported by a Wellcome Trust project grant to A.S., a KCL ORS award to L.L.-F., and MRC and Wellcome Trust project grants to C.H.

Received: August 11, 2012

Revised: February 20, 2013

Accepted: July 2, 2013

Published: July 29, 2013

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