RESEARCH NOTE

**xNgn2 induces expression of predominantly sensory neuron markers in *Xenopus* whole embryo ectoderm but induces mixed subtype expression in isolated ectoderm explants** [version 1; referees: awaiting peer review]

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Abstract

Proneural basic-helix-loop-helix (bHLH) proteins, such as Neurogenin2 (Ngn2) and Ascl1, are critical regulators at the onset of neuronal differentiation. Endogenously they have largely complementary expression patterns, and have conserved roles in the specification of distinct neuronal subtypes. In *Xenopus* embryos, xNgn2 is the master regulator of primary neurogenesis forming sensory, inter- and motor neurons within the neural plate, while xAscl1 is the master regulator of autonomic neurogenesis, forming noradrenergic neurons in the antero-ventral region of the embryo. Here we characterise neuronal subtype identity of neurons induced by xNgn2 in the ectoderm of whole *Xenopus* embryos in comparison with xAscl1, and in ectodermal “animal cap” explants. We find that the transcriptional cascades mediating primary and autonomic neuron formation are distinct, and while xNgn2 and xAscl1 can upregulate genes associated with a non-endogenous cascade, this expression is spatially restricted within the embryo. xNgn2 is more potent than xAscl1 at inducing primary neurogenesis as assayed by neural-β-tubulin. In ectoderm of the intact embryo, these induced primary neurons have sensory characteristics with no upregulation of motor neuron markers. In contrast, xNgn2 is able to up-regulate both sensory and motor neuron markers in naïve ectoderm of animal cap explants, suggesting a non-permissive environment for motor identity in the patterned ectoderm of the whole embryo.

Keywords

Neurogenin2, neurogenesis, *Xenopus*, bHLH, proneural, sensory, motor.
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Introduction
Proneural proteins are members of the basic-helix-loop-helix (bHLH) family of transcription factors, with a conserved role in driving and coordinating the various stages of neurogenesis\(^1\). In addition to neuronal specification, the concept of subtype determination by proneural factors was first established for achaete and atonal in Drosophila\(^2\). Vertebrate homologues Ascl1 and Neurogenin2 (Ngn2) also show largely complementary patterns of expression, with distinct roles in neuronal subtype and neurotransmitter phenotype throughout the nervous system\(^3\).

In Xenopus, xNgn2 (also known as X-ngnr-1) is the master regulator of primary neurogenesis\(^4\), during which neurons develop in the trigeminal ganglia and in three bilateral stripes within the neural plate to form sensory, inter- and motor neurons for coordination of larval reflexes\(^5\). This is analogous to the role of mammalian Ngn2 in specification of sensory and motor neurons in the dorsal root ganglia and ventral spinal cord, respectively\(^6\). Induction of primary neurons in Xenopus embryos by proneural transcription factors, identified by pan neuronal marker neuronal-\(\beta\)-tubulin\(^7\), is often used as a measure of proneural activity and activation of a generic neuronal pathway. What is less well documented is whether these induced neurons additionally acquire a subtype identity, and whether that identity differs depending on the inducing proneural factor.

In this short study, we focus on the key Xenopus proneural protein xNgn2 and define xNgn2 subtype specification during induced neurogenesis in the ectoderm of whole embryos and in isolated ectodermal “animal cap” explants.

Methods
Animal care
All work has been carried out under UK Home Office Licence and has passed an Institutional ethical review committee assessment at the University of Cambridge.

Plasmids and constructs
Wild-type Xenopus Ngn2 (Genbank accession number NM001088333) and wild-type Xenopus Ascl1 (Genbank accession number NM001085778) were subcloned into pCS2+ between BamH1 and Xho1 sites using primers:

\[
\begin{align*}
5\'_\text{BamH1}_x\text{Ngn2} &= \text{GATCGGATCCACATGTTCACTACATGTGCTGAT} \\
3\'_\text{Xho1}_x\text{Ngn2} &= \text{GATCCTCGAGTCAGAACCAAGTGGTGAAGTC} \\
5\'_\text{BamH1}_x\text{ash1} &= \text{GATCGGATCCACATGTTCACTACATGTGCTGAT} \\
3\'_\text{Xho1}_x\text{ash1} &= \text{GATCCTCGAGTCAGAACCAAGTGGTGAAGTC}
\end{align*}
\]

Xenopus laevis embryo manipulation
All efforts are made to ameliorate suffering to any animal. For example, the colony of approximately 80 X.laevis females are housed and cared for by a dedicated team of animal technicians operating under Home Office Licence. Each experiment requires eggs from 2 or 3 females (depending on N = 2 or 3) and females are used on rotation within the colony with at least a 3-month rest period after laying. A single male frog is sacrificed under humane conditions and Home Office Licence to provide testes for at least 16 experiments. Embryos obtained from fertilised eggs are used for the experiments and development is stopped 48 hours post fertilisation when embryos reach late neurula stage and prior to formation of tadpoles.

Thus, X. laevis eggs were obtained by standard hormone methods of induction, and subsequently fertilised \textit{in vitro}. pCS2+ constructs were linearised and capped mRNA was transcribed \textit{in vitro} using the SP6 mMessage mMachine\(^8\) kit (Ambion). Embryos were injected with mRNA as indicated in the \textit{Results} section, with GFP (for qPCR) or \(\beta\)-gal (ISH) as lineage tracers. Embryos were cultured at 18°C in Ficoll solution and staged according to 8. At stage 17–20, embryos were either snap-frozen for qPCR analysis or fixed in MEMFA for 90 minutes, as described in 9.

Whole mount \textit{in situ} hybridisation (ISH)
Dig-oxigennin-labelled anti-sense probes were synthesised from plasmids \textit{X.laevis neural-\(\beta\)-tubulin\(^7\)}, \textit{X.laevis xHox1L2\(^9\)}, \textit{X.laevis \textit{xHand2\(^a\)}} and \textit{X.laevis xPhox2a\(^a\)}\(^10\). Whole mount ISH was performed as described in 9.

Quantitative real-time PCR (qPCR)
GFP expression was used to confirm successful injection and samples of four embryos were snap frozen. Whole embryo RNA was extracted using the RNeasy\textsuperscript{\textregistered} Mini kit (Qiagen) and template cDNAs synthesised with the Quantitect\textsuperscript{\textregistered} Reverse Transcription Kit (Qiagen). qPCR was performed using the Quantifast\textsuperscript{\textregistered} SYBR Green PCR kit (Qiagen) in a LightCycler\textsuperscript{\textregistered} 480 (Roche). Thermal cycling conditions: 95°C for 5 minutes, then 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 20s. Primers provided in 14, see Data availability section for details.

Explant culture
For animal cap experiments, embryos were injected at the one cell stage and cultured in Ficoll solution at 14°C overnight. At stage 8, ectodermal explants from the animal pole of embryos (animal caps) were harvested in 1x MBS and subsequently cultured in 0.7x MBS at 14°C in 6-well plates with an agarose base, until corresponding whole embryos reached the desired stage. 18 caps per category were snap frozen for qPCR analysis.

Data analysis
For ISH images, representative images are shown from independent experiments and the N numbers reported refer to the total number of embryos per category. For qPCR data, mRNA expression was normalised to expression of reference gene (EF1\textalpha\textsuperscript{\textregistered} for whole embryos and ODC1 for animal caps) and mRNA levels in the injected embryos were calculated relative to stage-matched uninjected controls. Mean values are plotted and error bars show the standard error of the mean from N independent experiments. Statistical significance was calculated by a paired two-tailed student \(T\) test in Microsoft Excel; \(NS\) = not significant; \(*\) = \(p<0.05\); \(**\) = \(p<0.025\); \(***\) = \(p<0.0125\).
Results

Over-expression of xNgn2 induces primary neurons with sensory characteristics in the ectoderm of whole embryos

xNgn2 and downstream proneural protein xNeuroD4 (also known as Xath3) are endogenous mediators of the primary neuron cascade, with some homology to Drosophila atonal, and are capable of inducing lateral ectoderm tissue to express neuronal marker neural-β-tubulin. Previous work suggests that these ectopic neurons have characteristics of cranial sensory and Rohon-Beard sensory neurons, consistent with a report from over-expression of Ngn1 in zebrafish. In contrast, xAscl1 is normally transiently expressed in autonomic neuron precursors in the antero-ventral region of the embryo, and over-expression of xAscl1 leads to up-regulation of autonomic neuron markers over the ventral ectoderm.

In mammalian systems, Ngn2 is considered to have a permissive role in neuronal fate specification with subtype influenced by cellular context; for example, while Ascl1 mutant mice show defects in autonomic neurogenesis, if Ngn2 is expressed from the Ascl1 locus this defect can be partly rescued, and Ngn2 can promote autonomic rather than sensory identity in dissociated neural tubes in culture if BMP levels are elevated. To further explore induction of the primary and autonomic neuron cascades by xNgn2 in comparison to xAscl1 in Xenopus embryos, mRNA encoding either xNgn2 or xAscl1 were over-expressed in one cell stage embryos with subsequent analysis by qPCR at stage 17 (Figure 1A). xBrn3d and xHox11L2 were used as markers of sensory neurons; xVsx1 was used as an interneuron marker; xLim3 and xHb9 were used as motor neuron markers; xHand2 and xPhox2a were used as autonomic (noradrenergic) neuron markers; xPtf1a was used as an inhibitory (GABAergic) neuron marker.

xAscl1 is not a potent inducer of primary neurogenesis, and at this level of over-expression, xAscl1 achieves only a modest two-fold increase in N-β-tubulin (Figure 1A); higher doses of xAscl1 are required to induce ectopic expression more widely across the dorsal ectoderm (Figure 1C). In contrast to relatively weak N-β-tubulin induction, there is a substantial increase in sensory neuron markers, with a five-fold increase in xHand2 and a 30-fold increase in xPhox2a. In situ hybridisation (ISH) for xHand2 confirms this upregulation is in the antero-ventral region of the embryo (Figure 1D) and thus spatially distinct from dorsally located N-β-tubulin expression, demonstrating that these are separate and independent cascades restricted to specific regions of the embryo.

Compared to xAscl1, an equivalent 45pg dose of xNgn2 mRNA induces a much greater 10-fold increase in N-β-tubulin, accompanied by a 20-fold increase in both sensory neuron markers xBrn3d and xHox11L2 (Figure 1A). In contrast, minimal changes are seen in motor neuron markers xLim3 and xHb9, thus indicating that the induced N-β-tubulin positive neurons in the ectoderm are adopting a sensory identity, even though Ngn2 is required for both sensory and motor fate during normal development of the neural tube. Ectopic expression of both xNgn2 and xAscl1 result in prominent inhibition of the interneuron marker xVsx1, suggesting a re-specification of endogenous interneurons from the intermediate stripe on the neural plate; a finding noted previously for the interneuron marker xPax2. Focussing on autonomic neuron markers, while a 15-fold increase is seen in xPhox2a expression, there is only a small two-fold increase in xHand2 mRNA in response to ectopic xNgn2. Interestingly, ISH for xPhox2a expression (Figure 1E) reveals its up-regulation by xNgn2 is restricted ventrally in the embryo, thus as with xAscl1, the expression of these autonomic genes is spatially restricted from the primary neuron cascade. Furthermore, the relative potency of xNgn2 and xAscl1 in these two cascades reflects their endogenous functions; i.e. xNgn2 shows greater potency for induction of primary neurons while xAscl1 is more potent in the generation of autonomic neurons.

The general inability to induce expression of motor neuron genes in the lateral ectoderm has been reported previously, but may reflect a requirement for a higher level of xNgn2. Alternatively, motor marker up-regulation may occur at a later stage of development than previously assayed. Focussing on comparison between sensory versus motor neuron markers, the experiment was repeated assaying embryos at later stages of development (data from stage 20 embryos shown in Figure 1B) and testing a range of xNgn2 doses. With each two-fold increase in xNgn2 mRNA, there is a clear step-wise increase in N-β-tubulin expression, with a parallel increase in sensory neuron markers, but motor neuron markers do not increase even at high doses of xNgn2 mRNA. To compare the spatial pattern of distribution of ectopic neurons, stage 18 embryos were assayed by ISH for xHox11L2 and xHb9 as sensory and motor markers respectively (Figure 1F). xHb9 expression is confined to the neural tube region as in un.injected embryos, but the pattern of xHox11L2 expression mirrors the pattern of N-β-tubulin with extensive expression throughout the lateral epidermis outside the neural tube. Thus, in whole embryos, neurons induced ectopically by xNgn2 generally display sensory characteristics, while there is also some up-regulation of autonomic marker xPhox2a in the ventral epidermis, but ectopic motor neurons are not produced.

Over-expressed xNgn2 upregulates sensory and motor markers in animal cap explants

The relative lack of induction of ectopic motor neurons in the ectoderm region of whole embryos may be due to a non-permissive environment for this subtype cascade. This may occur if the endoderm contains an inhibitory signal, or lacks a necessary inductive signal; both of which may regulate epigenetic availability of promoters or necessary cofactors such as the LIM-HD proteins that are required for motor neuron identity in the mammalian ventral spine. To explore the activity of xNgn2 in a naïve environment in isolation from regional patterning or inductive signals in the developing embryo, we explored induction of neurons by xNgn2 in animal cap explants cultured in vitro.

Embryos were injected at the one cell stage with 45pg of xNgn2 mRNA, animal caps were excised from the roof of the
Figure 1. Over-expressed xNgn2 induces predominantly sensory neurogenesis in whole embryos. (A) One cell stage embryos were injected with 45pg mRNA encoding either xNgn2 or xAscl1. At stage 17, embryos were assayed by qPCR for expression of a range of neuronal subtype markers; [N=3]. Sensory neuron markers are induced by xNgn2 in parallel to the level of N-β-tubulin. xPhox2a is up-regulated but without induction of xHand2 that is co-expressed in autonomic neurons. (B) qPCR data from stage 20 embryos injected with increasing amounts of xNgn2 mRNA. [N=3]. A step-wise increase in N-β-tubulin is seen with parallel increase in sensory markers but no change in motor markers. (C–E) Embryos were unilaterally injected at the two-cell stage with mRNA as indicated and representative images of embryos at stage 18 are shown following in situ hybridisation; injected side to the right, stained with pale blue β-gal tracer. (C) N=49–61 embryos in each category in three independent experiments. DV, dorso-ventral view. xNgn2 is more potent than xAscl1 in inducing N-β-tubulin expression. (D) N=36–42 embryos in each category in two independent experiments. Endogenous xHand2 expression (white arrow) and induced xHand2 expression (black arrow) are confined to the antero-ventral region of the embryo. (E) N=31–39 embryos in each category in two independent experiments. Endogenous xPhox2a expression (black arrow) and induced xPhox2a expression (white arrow) are confined to the antero-ventral region of the embryo. (F) One cell stage embryos were injected with 45pg xNgn2 mRNA and representative images of embryos at stage 18 are shown following in situ hybridisation. N=21–26 embryos in each category in two independent experiments. The pattern of ectopic N-β-tubulin expression is mirrored by the pattern of ectopic xHox11L2 expression while there is no induction of motor marker xHb9 (white arrow).
blastocoel in stage 8 embryos and were then cultured until the corresponding whole embryos reached stage 17. Four independent repeat experiments were performed and in qPCR assays, N-β-tubulin and xBrn3d are found to be dramatically upregulated although the extent varies between experiments (Figure 2A), while additional markers are upregulated to a lower extent, replotted in Figure 2B using a different scale. In the naïve environment of animal caps, and in contrast to the results obtained with whole embryos, xNgn2 significantly upregulates a comparable seven to eight-fold increase in sensory marker xHox11L2 and motor marker xHb9, while trends indicating four to five-fold increases in xLim3 and interneuron marker xVsx1 are just outside statistical significance. A similar increase is seen in xPhox2a but induction of xHand2 is not observed. Taken together, naïve caps upregulate a mix of all three primary neurons subtypes (motor, sensory and interneuron) in response to ectopic xNgn2 expression.

Animal caps expressing xNgn2 were also assayed for expression of regional neural markers using Otx2 as a fore/midbrain marker28, En2 as a caudal midbrain marker29, Krox20 as a hindbrain marker30 and XlHbox6 as a posterior neural plate marker31. Small changes in expression of regional markers may indicate a trend towards more anterior CNS than posterior neural plate identity but statistical significance of any changes compared to naïve caps is not reached, so firm conclusions cannot be drawn.

Figure 2. Over-expressed xNgn2 upregulates sensory and motor markers in animal cap explants. One cell stage embryos were injected with 45pg mRNA encoding xNgn2. At stage 8 animal caps were cut and cultured until the respective whole embryos were at stage 17 (A, B) or stage 20 (C), and assayed for gene expression by qPCR, relative to uninjected naïve caps. (A) Gene expression for the full range of subtype markers; N-β-Tubulin and xBrn3d are dramatically up-regulated but variable in absolute magnitude in the cap assays [n=4]. (B) Data replotted without N-β-Tubulin and xBrn3d. Statistical significance was calculated relative to naïve caps by a paired two-tailed student T test; NS, not significant; * = p<0.05; *** = p<0.0125. In contrast to whole embryos, xNgn2 upregulates motor neuron markers in animal caps to an equivalent level as sensory marker xHox11L2. (C) Expression of regional markers show a trend to more anterior rather than posterior neuronal identity [N=2].
Conclusions
In this short study we have characterised xNgn2 proneural activity in the ectoderm of whole embryos in comparison to the activity of xAscl1, as well as its activity in isolated ectodermal animal cap explants. In whole embryos, ectopic primary neurons generated show predominantly sensory characteristics with the pattern of sensory marker xHox11L2 mirroring that of N-β-tubulin. Mammalian Ngn2 has a critical and specific role in sensory neurogenesis that cannot be rescued by Ascl1, but over-expression of Ngn2 in chick embryos does not up-regulate a full complement of sensory markers; thus more detailed analysis, for instance by transcriptome-wide comparison with neurons from a normal developing embryo, is required to determine the extent of sensory reprogramming.

xNgn2 and xAscl1 are the respective master transcriptional regulators of primary and autonomic neurons in Xenopus, and while both are able to upregulate markers from the alternative cascade, in whole embryos this upregulation is spatially confined; primary neurogenesis dorsally and autonomic neurogenesis ventrally. This is reminiscent of that reported in Drosophila whereby atonal promotes formation of external sense organs but also generates spatially restricted ectopic chordotonal organs. Again, it is currently unclear if this represents full activation of the non-endogenous cascade or perhaps a redundant function at only specific downstream targets that are more easily activated due to permissive chromatin in that anatomical region of the embryo.

In contrast to the marked lack of motor neuron gene up-regulation and the dramatic inhibition of interneuron marker in whole embryos, xNgn2 upregulates all three primary neuron subtypes in naïve ectoderm of animal caps. This indicates that patterning factors or signals in the ectoderm of the intact embryo are non-permissive for the motor neuron pathway and may suggest why reprogramming approaches for human patient-derived motor neurons from fibroblasts have required an induced pluripotent stage to erase somatic cell identity. Further exploration using the whole embryo and explan assays in Xenopus may help to elucidate the patterning factors and molecular determinants required for efficient motor neuron specification in the reprogramming environment.

Data availability

See Methods section for description of data analysis and ISH scoring. Datasets presented are as follows:
- Fig1A_qPCR and Fig1B_qPCR: Mean fold change in expression for each target gene relative to uninjected controls; three independent experiments.
- Fig1C_embryos: Representative images from 49–61 embryos in each category from three independent experiments.
- Fig1D_embryos: Representative images from 36–42 embryos in each category in two independent experiments.
- Fig1E_embryos: Representative images from 31–39 embryos in each category in two independent experiments.
- Fig1F_embryos: Representative images from 21–26 embryos in each category in two independent experiments.
- Fig2A+B_qPCR: Mean fold change in expression for each target gene relative to uninjected naïve caps; four independent experiments.
- Fig2C_qPCR: Mean fold change in expression for each target gene relative to uninjected naïve caps; two independent experiments.
- Primers_qPCR: Primers used for qPCR analysis.

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References
XHox11L2 expression is restricted to a subset of the primary sensory neurons. Dev. Dyn. 1999; 214(1): 34–43.


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