Neural abnormalities in insulin-deprived frog embryos are associated with altered gene expression

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Insulin plays an essential role in the prepancreatic embryonic development of the frog, *Microhyla ornata*. While exogenous insulin stimulates neurulation as well as overall embryonic development in this organism, immunoneutralization of endogenous insulin leads to peculiar abnormalities in the developing nervous system. In the present report, we demonstrate that neural abnormalities in embryos treated with antiserum to insulin are associated with altered gene expression. By using whole-mount *in situ* hybridization, we show that the expression of *noggin*, the product of which is involved in dorsalization and neural induction, is altered in such embryos concomitant with abnormal nervous system development. The action of exogenous insulin also seems to be mediated through crucial developmental genes such as *Brachyury* and *noggin*. This report demonstrates that developmental gene expression is altered in frog embryos developing under conditions of excess/scarcity of insulin.

INSULIN, insulin-like molecules and insulin signalling are involved in a variety of development-related phenomena in invertebrates and vertebrates[1–11]. We have shown earlier that insulin plays an essential role in the prepancreatic development of the frog *Microhyla ornata*[5,6]. Exogenous, porcine insulin stimulates the process of neurulation as well as overall embryonic development in this frog. *Microhyla* embryos, in which endogenous insulin is neutralized with antiserum to insulin, exhibit development of an abnormal nervous system. The abnormalities include aberrant architecture and reduction in the size of the neural tube and rotation of the dorso-ventral axis of the neural tube along with the adjoining mesodermal structures such as the notochord and somites[6]. In recent years, it has been amply demonstrated that early embryonic development and pattern formation are driven and controlled by specific genes. The correct temporal and spatial distribution of transcripts of such genes is necessary for normal development; this is largely governed by cytoplasmic determinants and signalling peptides[12–16]. In view of the widespread involvement of insulin in early animal development mentioned above, it is necessary to examine the possibility of insulin exerting its action through genes crucial for normal development. To our knowledge, there has been no attempt so far in this direction, except for the reported association of reduced *Pax-3* expression with neural tube defects, in embryos of diabetic mice[17].

The present study was initiated to examine the effects of experimentally created scarcity of insulin on the expression of *noggin*. *Noggin* is localized in the Spemann's organizer in *Xenopus* embryo and its product is a secreted protein that participates in dorsalization of mesoderm and neural induction[18]. Over-expression of *noggin* transcripts at ectopic sites causes dorsalization, while in ventralized embryos, it rescues the normal phenotype[18]. Our previous work with *Microhyla* embryos[5,6] has shown that immunoneutralization of endogenous insulin leads to the development of an abnormal nervous system. Although the molecular cascade leading to induction and pattern formation of nervous system would necessarily involve a large number of genes, we started by looking at the expression of *noggin* since it is one of the most crucial components of this pathway (reviewed in refs 19, 20). We have also looked at the modulation of embryonic gene expression by excessive insulin. This would tell us if normal functioning of insulin involves development-regulating genes. Here, along with *noggin*, we have also looked at the expression of *Brachyury*. The *Xenopus Brachyury*, is a pan-mesodermal marker[21] gene that codes for a transcription factor[22] and controls the expression of several downstream genes required for normal mesodermal development[21,23,24] (reviewed in refs 25, 26). We have studied the expression of this gene in the presence of excess insulin, since it regulates the development of notochord that, in turn, generates neural inducing signals. Moreover, an interaction between *Brachyury* and *noggin* is necessary for the dorsalizating action of the latter in *Xenopus* animal caps[27]. However, in a developing embryo, the action of *Brachyury* is thereafter restricted to the development of the posterior mesoderm. Therefore, *Brachyury* expression was not studied under conditions of scarcity of insulin, since these studies were carried out on developmentally advanced embryos (early neurula, stage 16)[28]. Data obtained in the present study demonstrate that neural abnormalities in insulin-deprived frog embryos are indeed associated with altered *noggin* expression. Further, exogenous insulin modulates the expression of *Brachyury* and *noggin* in developing frog embryos.

Freshly fertilized eggs of the frog *M. ornata* were collected from natural ponds and allowed to develop at room temperature (24–26°C) till the desired stage of development. The embryos were dejellyed with 2% cysteine, pH 8.9 and treated as described before[5,29]. Early neurula (stage 16)[28] stage embryos were treated with antiserum to porcine insulin (final dilution 1 : 500) for 4, 6 or 18 h. The corresponding controls received an identical dilution of pre-immune goat serum. In the case of treatment with insulin, early gastrula (dorsal lip of blastopore, stage 9)[28] or early neurula (stage 16)[28]

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embryos were treated with 100 µg/ml porcine insulin for 3 h. The corresponding controls received equal amount of filtered and sterile pond water. The concentrations of insulin and antiserum to insulin were used based on earlier studies[5,6].

Xenopus noggin (*X*-nog)[18] and *Xenopus Brachyury* (*X*-bra)[30] cDNAs cloned in pGEM5Zf(–) and pSP73 vectors, respectively, were a kind gift from J. C. Smith, Cambridge. The plasmid *pnoggin5.5* was linearized with *Eco*RI and transcribed using T7 RNA polymerase in the presence of DIG-labelled dUTPs to get antisense transcript of 463 bases. Likewise, DIG-labelled *X-Bra* probe (2299 bases) was generated by using T7 RNA polymerase from the plasmid pXT1 linearized with *Eco*RV.

At the end of the treatment period, the embryos were processed for whole-mount *in situ* hybridization according to the method of Harland[31] with a few modifications. The embryos were fixed in 4% paraformaldehyde at 4°C overnight, washed in PTw (1X PBS plus 0.1% Tween-20) and treated with Proteinase K (10 µg/ml, 30 min, room temperature), with occasional shaking. After washing in PTw, embryos were refixed in 4% paraformaldehyde (in PTw) at 4°C for 20 min. Prehybridization was carried out at 60°C for 4–6 h followed by hybridization at 60°C overnight. The detection was carried out with anti-DIG antibody coupled to alkaline phosphatase and by using BCIP and NBT (Roche Molecular Biochemicals). Embryos were fixed in Bouins fixative (4°C, overnight), washed thoroughly with PBS, bleached in bleaching solution (70% methanol, 30% H_2O_2) at room temperature overnight under strong fluorescent light (tubelight), washed in methanol, rehydrated, mounted in 50% glycerol and photographed under incident light.

M. ornata embryos treated with antiserum to insulin at the beginning of neurulation for 4, 6 or 18 h at room temperature (24–26°C) were processed for *in situ* hybridization. In the control embryos, developing in the presence of preimmune serum for 4 h, *noggin* expression was localized all along the neural groove (Figure 1 *a*). The approaching edges of the neural folds were sharply defined by discrete staining for *noggin*. The anterior-most tip of the neural folds was more strongly stained than the remaining parts (Figure 1 *a*). The overall localization of *noggin* in the embryos treated with antiserum to insulin for 4 h was similar to that of controls except that the expression was diffused all along the anteroposterior axis (Figure 1 *b*). At 6 h, the differences between the controls and treated embryos became more apparent (Figure 1 *c*, *d*). In the controls (Figure 1 *c*), *noggin* transcripts were visible mainly in the anterior one-third of the mid-dorsal line. The expression of *noggin* disappeared from the remaining two-third of the mid-dorsal line, presumably after the closure of the neural tube. In contrast to this, *noggin* expression persisted throughout the mid-dorsal line in embryos treated with anti-insulin antiserum (Figure 1 *d*). The lengthening of the anteroposterior axis was much more in control embryos (Figure 1 *c*) than in the treated embryos (Figure 1 *d*). This, together with the *noggin* expression, clearly shows that the embryos treated with antiserum to insulin are already discernibly retarded in development at 6 h of treatment.

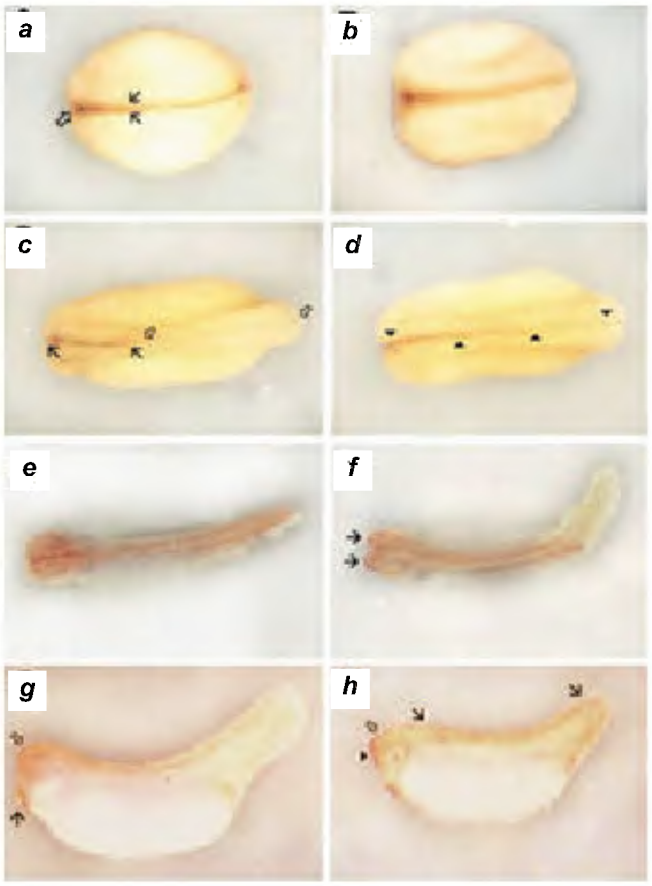

Figure 1. Expression of *noggin* in *Microhyla ornata* embryos treated with antiserum to insulin (1 : 500) at the early neurula stage. ***a***, Dorsal view of control embryo treated with preimmune goat serum (1 : 500) for 4 h. Note expression of *noggin* along the anteroposterior axis. Prominent staining can be seen in the anterior-most end (hollow arrow) as well as at the approaching edges of the neural folds (arrows); ***b***, Dorsal view of a corresponding embryo treated with antiserum to insulin for 4 h. Note slight reduction in the staining intensity. The staining along the neural folds appears diffused compared to that seen in the control; ***c***, Dorsal view of a control embryo at 6 h of treatment. Note *noggin* expression only in the anterior one-third along the anteroposterior axis (arrows). The expression from the posterior two-thirds has disappeared (hollow arrows); ***d***, Dorsal view of an embryo treated with antiserum to insulin for 6 h. Note persisting *noggin* expression all along the anteroposterior axis (arrowheads). This is associated with retardation of elongation of the anteroposterior axis as seen after comparing the elongation of axis in control and treated embryos; ***e***, Dorsal view of the control embryo 18 h post-treatment. *Noggin* expression is seen in the brain region as well as all along the anteroposterior axis; ***f***, Dorsal view of a corresponding treated embryo. Note slightly reduced expression in the brain region associated with microcephaly, due to which cement glands (arrows), located ventrally, have become visible in the dorsal view; ***g***, Lateral view of a control embryo 18 h post-treatment. Note normal development of brain (hollow arrow). Cement glands (arrow) also show *noggin* expression; ***h***, Lateral view of a corresponding treated embryo. Note mocrocephaly (hollow arrow) associated with persistent *noggin* expression in the anterior-most tip of brain (arrowhead) and along the anteroposterior axis (arrows).

Retarded development associated with altered *noggin* expression due to scarcity of insulin became clearly apparent after 18 h (Figure 1 *e–h*). In the control embryos, *noggin* transcripts were localized throughout the mid-dorsal line, with prominent staining in the brain region (Figure 1 *e*). There was an overall reduction in *noggin* staining in the treated embryos, which was particularly evident in the head region (Figure 1 *f*). It is interesting to note that this reduction in *noggin* in embryos treated with antiserum to insulin is associated with microcephaly, as a result of which cement glands are visible from the dorsal side (Figure 1 *f*). Normally, the cement glands which are located ventrally are not visible in a dorsal view (Figure 1 *e*). Microcephaly and retardation in the elongation of the anteroposterior body axis can be more clearly seen in the lateral view (Figure 1 *g*, *h*). These morphologically distinct differences are also reflected in *noggin* expression. *Noggin* expression still persists along the anteroposterior axis in the treated embryo (Figure 1 *h*) unlike in the controls (Figure 1 *g*). A more prominent staining for *noggin* is seen in the anterior-most tip of the treated embryos concomitant with incomplete brain development (Figure 1 *h*).

Analysis of *noggin* expression in embryos developing in the presence of antiserum to insulin demonstrates that experimentally created scarcity of endogenous insulin leads to altered *noggin* expression. This is associated with abnormal brain development. A direct connection between altered *noggin* expression and abnormal neural development cannot be concluded from the available data. However, in view of the crucial role *noggin* plays in patterning of the mesoderm (dorsalization) and induction of the nervous system[18–20], it appears likely that alteration in its expression is at least partially responsible for the observed phenotype. It is likely that several other genes are also involved in this process; this needs to be studied. In any case, the present results provide evidence for modulation of gene expression in response to scarcity of insulin and its close association with neural anomalies.

The expression of *Brachyury* was found all over the blastoporal lips of the control embryos (Figure 2 *a*). This was found to be significantly reduced in embryos treated with exogenous insulin (Figure 2 *b*). Even in the embryos treated at the early neurula stage, *Brachyury* expression was found to be reduced (Figure 2 *c*, *d*). *Brachyury* expression seen along the approaching neural folds (Figure 2 *c*) was significantly reduced due to insulin treatment (Figure 2 *d*). The functional significance of this reduction in *Brachyury* expression due to insulin is not clear at this stage.

In the control embryos, *noggin* transcripts were detected in the neural plate and the neural folds (Figure 2 *e*). In insulin-treated embryos, there was a significant reduction in *noggin* expression in the neural plate (Figure 2 *f*).

The results of this set of experiments demonstrate that exogenous insulin can modulate the expression of at least two genes crucial for normal development and pattern formation. The significance of reduction in the expression of *Brachyury* and *noggin* in response to insulin treatment, in the context of progression of development, is not clear at this stage. The two genes have multiple downstream targets important in pattern formation. Continued sub-optimal expression of *Brachyury* and *noggin* in insulin-treated embryos is likely to have some effect on the final outcome of development. This possibility is yet to be addressed. A curious observation in the present study was that the expression of *noggin* is down-regulated under conditions of excess insulin as well as scarcity of insulin.

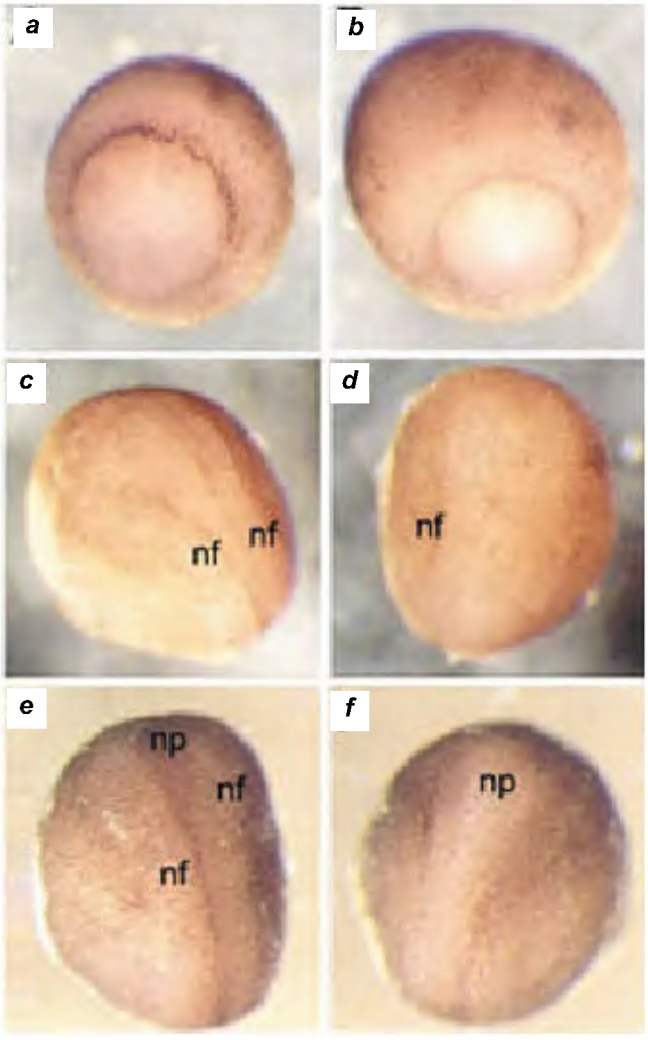

Figure 2. Modulation of expression of *Brachyury* and *noggin* in *Microhyla* embryos due to treatment with exogenous insulin (100 µg/ml) for 3 h. ***a***, Control embryo showing *Brachyury* expression around the rim of the blastopore; ***b***, Corresponding embryo treated with insulin at dorsal lip of blastopore stage. Note overall reduction in the staining for *Brachyury*; ***c***, Control embryo showing *Brachyury* expression in the neural folds (nf); ***d***, Corresponding embryo treated with insulin at early neurula stage. Note overall reduction in the staining for *Brachyury*; ***e***, Control embryo showing expression of *noggin* in the neural folds (nf) and the neural plate (np); ***f***, Corresponding embryo treated with insulin at early neurula stage. Note significant reduction of *noggin* in the neural plate (np).

The reason for this is not known at this moment, but it could be related to different regulatory mechanisms controlling the spatiotemporal expression of *noggin*, depending on its role and site of expression at different developmental stages, as is the case with chick embryos[32,33].

Insulin has been shown to be present in the neurulating *Xenopus* embryos[34,35]. Similarly, genes encoding receptors for insulin have also been shown to be expressed in *Xenopus* embryos[36]. We have earlier demonstrated that excess insulin stimulates amphibian embryonic development while scarcity of insulin leads to abnormal development, especially of the nervous system[5,6]. Data obtained in the present study show that (1) exogenous insulin can modulate the expression of genes in the developing embryos, and (2) neural abnormalities in insulin-deprived embryos are associated with altered expression of *noggin*. Taken together, these results show that the role of insulin in prepancreatic embryonic development involves modulation of at least some genes important in development. Since most of such genes are highly conserved in the animal kingdom, these findings may explain the development-related role of insulin in diverse organisms such as *C. elegans*[10], *Drosophila*[8,9], locust[7], silkworm[11], sea urchin[1], frog[5,6], chick[2] and mammals[37].

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