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Revisión

Old hormones of the insulin family as new developmental signals

FLORA DE PABLO[*, 1], CATALINA HERNÁNDEZ-SÁNCHEZ[1], CARLOS VICARIO-ABEJÓN[1, 2] AND ENRIQUE J. DE LA ROSA[1]

[1] *Group of Growth Factors in Vertebrate Development, Centro de Investigaciones Biológicas (CSIC, Madrid).*

[2] *Instituto Cajal (CSIC, Madrid)*

ABSTRACT

Insulin was first identified as an anabolic pancreatic hormone responsible for glucose homeostasis, and Insulin-like Growth Factor (IGF-I) as the mediator of the action of Growth Hormone on postnatal growth. New molecular, pharmacological and embryological information has broadened the scope of the physiological roles of these hormones and their related molecules, particularly the insulin precursor proinsulin, during vertebrate development. Studies in our laboratory have demonstrated that proinsulin is expressed and functional before emergence of the pancreas. Proinsulin gene expression in the chick and mouse embryo shows fine transcriptional and postrancriptional regulation with generation of specific embryonic transcripts which are differentially translated. The protein product remains as unprocessed proinsulin that protects the cells from excessive apoptosis during neurulation. In contrast, IGF-I is expressed later than proinsulin in the chick embryo and it starts in the nervous system. In the mouse embryo, generation of olfactory bulb stem cells in culture has allowed the study of these molecules' role in the proliferation and differentiation of neural precursors. Proinsulin and IGF-I can cooperate with mitogens (EGF and FGF2) in the control of stem/precursor cells proliferation and IGF-I is an essential factor for neural differentiation. Mice deficient in IGF-I present a disruption of olfactory bulb cytoarchitecture, with decreased numbers of mitral cells and abnormal radial glia. This article gives thus an overview of the important role of insulin family proteins in development.

* Toma de Posesión como Académica Correspondiente, 4 de noviembre de 2004.

Key words: Proinsulin.—Insulin-like growth factor I.—Embryo.— Stem cells.—Neurogenesis.

RESUMEN

Hormonas tradicionales de la familia de la insulina como nuevas señales en el desarrollo

La insulina fue identificada como una hormona anabólica pancreática, responsable de la homeostasis de la glucosa, y el Factor de Crecimiento similar a la Insulina tipo I (IGF-I) como el mediador de la acción de la Hormona de Crecimiento postnatalmente. Nuevas informaciones moleculares, farmacológicas y embriológicas han ampliado el concepto del papel fisiológico de estas hormonas y sus moléculas relacionadas, particularmente del precursor de la insulina, la proinsulina, en el desarrollo de vertebrados. Los estudios de nuestro laboratorio han demostrado que la proinsulina está expresada y es funcional antes de que aparezca el páncreas. La expresión de proinsulina en los embriones de pollo y ratón muestra regulación transcripcional y post-transcripcional muy fina, con la generación de transcritos específicos embrionarios que se traducen de formas distintas. El producto de estos mRNAs se mantiene como proinsulina sin procesar, que protege a las células de la apoptosis excesiva durante la neurulación. En contraste, el IGF-I está expresado más tarde que la proinsulina en el embrión de pollo y comienza en el sistema nervioso. En el embrión de ratón, la generación de células madre neurales en cultivo ha permitido estudiar el papel de estas moléculas en la proliferación y diferenciación de precursores neurales. La proinsulina y el IGF-I pueden cooperar con los mitógenos (EGF y FGF2) en el control de la proliferación de células madre/precursores mientras que el IGF-I es un factor esencial para la diferenciación neural. Los ratones deficientes en IGF-I presentan alteración de la citoarquitectura del bulbo olfatorio con disminución del número de neuronas mitrales y glía radial anormal. Este artículo da una visión global del importante papel de las proteínas de la familia de la insulina en el desarrollo.

Palabras clave: Proinsulina.—Factor de crecimiento similar a la insulina tipo I.— Embrión.—Células madre.—Neurogénesis.

INTRODUCTION

We celebrate this year, 2005, the 100th anniversary of the birth of Endocrinology as a discipline, marked by the introduction of the term «hormone» by Ernest Henry Starling. He proposed this name to designate *certain chemicals, elaborated by glands and*

transported to distant body parts through the bloodstream, modifying body functions (38). In the following decades, the study of hormones was thus parallel to the study of the so named endocrine glands, thinking that they were *fixed couples*. With the speed of biological discovery in the «omics» era (genomics, proteomics, metabolomics, etc.), however, very few concepts have the label of *dogma* for very long time.

Insulin, since it was discovered by Banting and Best in 1921 and extracted from the dog pancreas (2), started a medical and pharmaceutical revolution in the treatment of diabetics, and remained known as an exclusive pancreatic hormone of vertebrates for most of the 20th century. With the advance of molecular and cellular techniques in recent years, it has become evident that insulin and its precursor proinsulin are expressed prior to pancreatic development and in organisms without a pancreas. In essence, all multicellular organisms are thought to have ancestral or evolved protein(s) of the insulin family (6) whereas the yeast genome does not contain any similar protein (8). The protochordate Amphioxus contains a single proinsulin-like gene (7) and more recent acquisitions led to the appearance of insulin-like growth factor (IGF) I and II in fish, amphibians and birds (6). In humans, there is an extended insulin family of proteins including relaxin, placentin and Leydig insulin-like peptide (21). These peptides signal the cells binding to several types of membrane tyrosine kinase receptors, from the ancestral single insulin receptor found in Amphioxus (30), to the mammalian diversified forms. These include the two forms of insulin receptor, containing or skiping exon 11 (4), the IGF-I receptor (15) and the hybrid receptors generated by them (17,29). Autophosphorylation of tyrosine residues in the ß-subunit of these receptors leads to phosphorylation in insulin receptor substrates (IRS) 1 to 4 and other transduction molecules (26,34), activating two major intracellular pathways: the PI3-kinase/Akt and the Ras/MAPK pathways (5,24). These signalling cascades and networks of activated molecules influence multiple cellular processes, including proliferation, cell growth, differentiation, migration, metabolism, and overall life span (16,20,33).

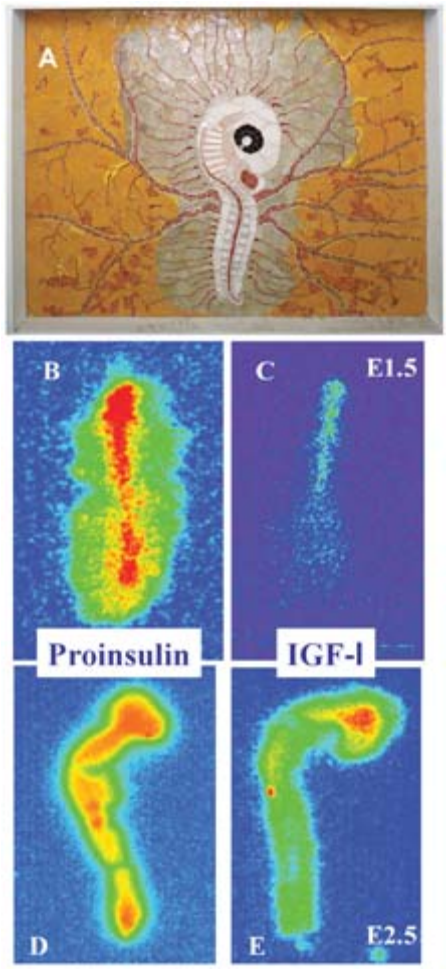

FIGURE 1. *A) Mosaic representing a chick embryo of approximately 3 days of development (E3) (Photography courtesy of Prof. Nicole Le Douarin. Institut d'Embriologie Cellulaire et Moléculaire, Nogent-sur-Marne, France). B) and D) In situ hybridization to demonstrate proinsulin mRNA distribution in the E1.5 and E2.5 chick embryo respectively. The image is processed in pseudocolor, red indicates high signal. C) and E) In situ hybridization for IGF-I mRNA at the indicated days of development (25).*

Expression of proinsulin and IGFs in chick embryo gastrulation and neurulation

The chick embryo has been a classic vertebrate model to analyze development from gastrulation, when the three embryo layers are defined, to neurulation and early organogenesis. It is accessible and indeed a beautiful artistic inspiration (Fig. 1A). Despite the lack of a pancreatic rudiment morphologically until the third day of incubation (E3), we found immunoreactive proinsulin/insulin in extracts of E2 embryos by radioimmunoassay (11). The possibility of quantifying the low amounts, as compared to pancreas, of embryonic proinsulin mRNA was possible only by using large amounts of Poly-A^+ RNA, but confirmed the presence of transcripts in E2 chick embryos (36). Surprisingly, even though IGF-I had been recognized as a broadly expressed tissue growth factor, IGF-I mRNA was minimally expressed prior to E3 in chick embryos, even when analyzed by the highly sensitive technique of reverse transcription coupled to polymerase chain reaction (RT-PCR) (37). Indeed, we could confirm both by RT-PCR (31) and by in situ hybridization (Fig. 1) that during neurulation proinsulin mRNA was broadly expressed along the embryo whereas IGF-I was barely detectable (Fig. 1B, C) (25). In early organogenesis, in E2.5 embryos, the expression of proinsulin mRNA was more restricted, but still significant in developing brain, and the two pancreatic buds (which will later be fused) were also positive (Fig. 1D). IGF-I initial expression was mostly localized in cephalic vesicles in E2.5 (Fig. 1E).

We were interested in defining if the protein produced by this proinsulin transcript was unprocessed proinsulin or mature insulin and for that purpose we developed an antiserum against the connective C-peptide that is cleaved during processing of proinsulin by the specific convertases. Immunofluorescence performed with this antiserum confirmed the presence of proinsulin in distinct cells of the three embryonic layers by E1. A few neuroepithelial cells were the most clearly labelled (Fig. 2B). This was in contrast with the absence of IGF-I in parallel sections (Fig. 2C), which agreed with the previous RNA data. IGF-II was found in extracellular, likely basal membrane, locations (Fig. 2D). With further development, in E2.5, at mid embryo level, multiple structures expressed proinsulin,

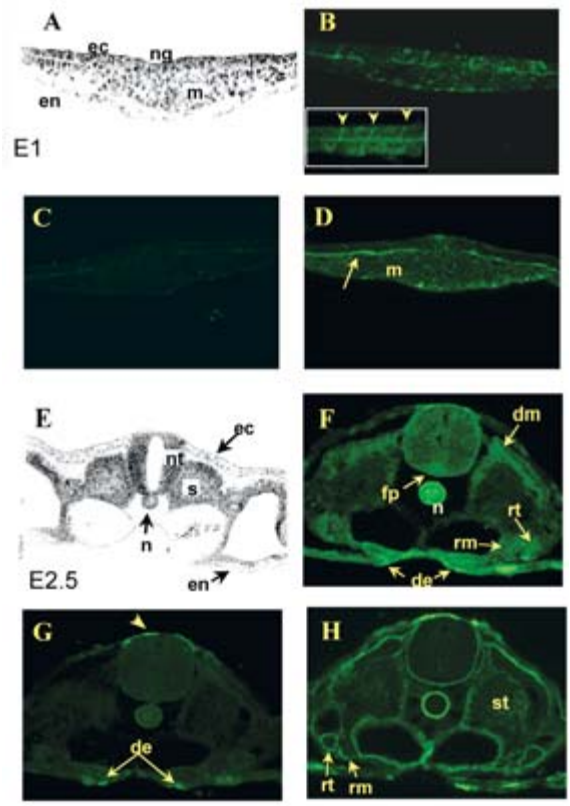

FIGURE 2. *Distribution of factors by immunofluorescence in chick embryos. A-D) E1 embryos (stage 6), E-H) E2.5 embryos (stage 15). Transversal criosections at mid-level in A, and caudal to the viteline artery in E (microscopy taken from Bellairs and Osmond, The Atlas of Chick Development. 1998. San Diego, California, Academic Press). Proinsulin is detected with an antipeptide-C antiserum (Rb40) in B (inset shows a detail with labelled neuroectodermic cells in the lateral zone, arrowheads) and F (positive areas include the floor plate of the neural tube, notochord and dermamyotome of somite). IGF-I immunostain is negative in C and labelled only a few ectodermic (short arrow) and digestive endodermic cells (long arrows) in G. IGF-II immunostain labelled basal membranes in D (arrow) and H (note around notochord and neural tube). Fluorochorome was Cy2 and images were captured with a CCD camera. ec, ectoderm; m, mesoderm; en, endoderm; n, notochord; ng, neural groove; nt, neural tube; s, somite; fp, floor plate; dm, dermomyotome; st, sclerotome; de, digestive endoderm; rm, renal mesenchyme; rt, renal tubule.*

including the floor plate of the neural tube, notochord, dermomyotome of the somite, and cells in the rudiment of the renal tubule and

digestive endoderm (Fig. 2F). IGF-I protein was detected in a few ectoderm cells in the dorsal part of the neural tube and a few cells in the digestive endoderm (Fig. 2G). IGF-II was more broadly present, decorating multiple basal membranes (Fig. 2H).

Overall, members of the insulin family are therefore present at very early stages of vertebrate development, and the prohormone proinsulin, previously believed to be nearly inactive, was expressed in a tissue- and time-specific manner in the chick embryo. We had proposed that there was an *endocrinization* of the early embryo (14), but these hormone-like factors were there before circulation was established, thus acting in more of a short-range, *paracrine* or *autocrine* manner, rather than in the canonical endocrine, longe-range form.

Embryonic proinsulin transcripts: more surprises

The multiple studies performed on pancreatic insulin regulation aimed at understanding the physiopathology of diabetes and approaches for therapy had demonstrated that glucose was the major stimulus for proinsulin gene expression and insulin secretion (41). Our studies with cultured prepancreatic chick embryos showed that proinsulin mRNA at that stage was not regulated by glucose (31). The analysis of the embryonic transcript provided further surprises, since at least two different embryonic mRNAs were identified, distinct from pancreatic proinsulin mRNA.

The chick embryo contains a single proinsulin gene, as in the human, and in contrast to the mouse, in which the proinsulin gene has been duplicated (see later). The protein coding region is contained in exon 2 and exon 3, whereas exon 1 is untranslated (5'UTR). The product obtained by rapid amplification of the cDNA end (5'RACE) was different in the pancreas than in the E1.5 chick embryo (Fig. 3).

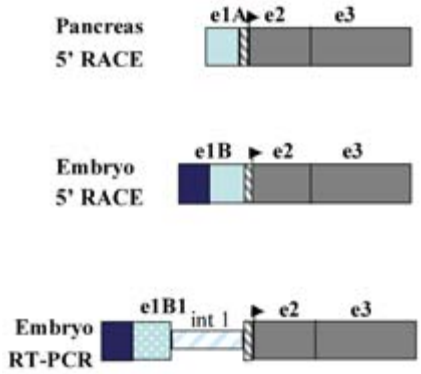

FIGURE 3. *Schematic representation of different embryonic and pancreatic proinsulin mRNA transcripts. Exons (e) and intron 1 (int) are represented. The arrow above indicates the translation initiation site. The techniques used were 5'RACE and RT-PCR with total RNA from pancreas and E1.5 chick embryos.*

Exon 1 of the embryo had an extension of 32 nucleotides with respect to exon 1 of the pancreas (19) yielding two mRNAs that we termed Pro1B and Pro1A, respectively. The protein product in both cases was proinsulin. Translation of the embryonic form is markedly downregulated by the presence of two upstream AUGs within the 32 nucleotide extension of the Pro1B mRNA (19). A later approach using PCR confirmed the expression from gastrulation to organogenesis of the Pro1B transcript, and revealed another alternative proinsulin transcript, a form that retained intron 1 (717 nucleotides) (28) (Fig. 3). Termed Pro1B1, it is a mature cytoplasmic mRNA but its translational capacity is nearly blocked due to its long leader region. There was differential developmental regulation of this new mRNA, with increasing abundance from gastrulation to neurulation and organogenesis.

We wonder whether this highly sophisticated regulation of proinsulin expression had any possible physiological implication and tried to look at events in early development that may be modulated by insulin or proinsulin.

Abnormal levels of proinsulin/insulin signalling cause changes in programmed cell death

Since classic binding-competition and autoradiography studies had revealed that the chick embryo had insulin receptors and IGF-I receptors from the stage of gastrulation onwards (1,18), we suspected that the proteins of the family had previously unknown roles in early development. We focused our attention on the process of programmed cell death, generally occurring as apoptosis, which is relevant to multiple morphogenetic events in development (43) including the closure of the neural tube (Fig. 4A). Using the technique of Terminal Deoxynucleotidyltransferase-mediated UTP Nick End Labelling (TUNEL), we could quantify the number of cells of a chick embryo during neurulation that undergo apoptosis, in vivo and in culture (Fig. 4B, C). When the embryonic endogenous proinsulin signal was interfered by using antisense oligonucleotides against proinsulin mRNA (25) or the insulin receptor mRNA (19), there was an increase in the number of cells dying of apoptosis (Fig. 4). Preventing cell death, thus, appeared as an important function of embryonic proinsulin. The modulation of the chaperone heat shock cognate 70 by proinsulin/insulin was found to correlate with the prevention of apoptosis (10).

We had known for some years that correct proinsulin/insulin signalling was needed to continue normal development beyond neurulation since insulin or insulin receptor antibodies caused a certain level of malformed or dead embryos between E2 and E4 of development (12). Opposite pharmacological experiments involving the addition of excess insulin or proinsulin also caused abnormalities and death (13).

In a similar way, it was found critical during neurulation to maintain the proinsulin signal within physiological limits. An excess of exogenously added proinsulin caused abnormalities that affected the neuropore, neural tube, and optic vesicles in the neurulating embryo (Table I), concomitant with a decrease in natural apoptosis (42). A tightly regulated proinsulin gene expression, therefore, is required to maintain the level of cell survival/cell death also strictly regulated in the embryo. This suggests an early important role for a protein prohormone previously thought to have little activity.

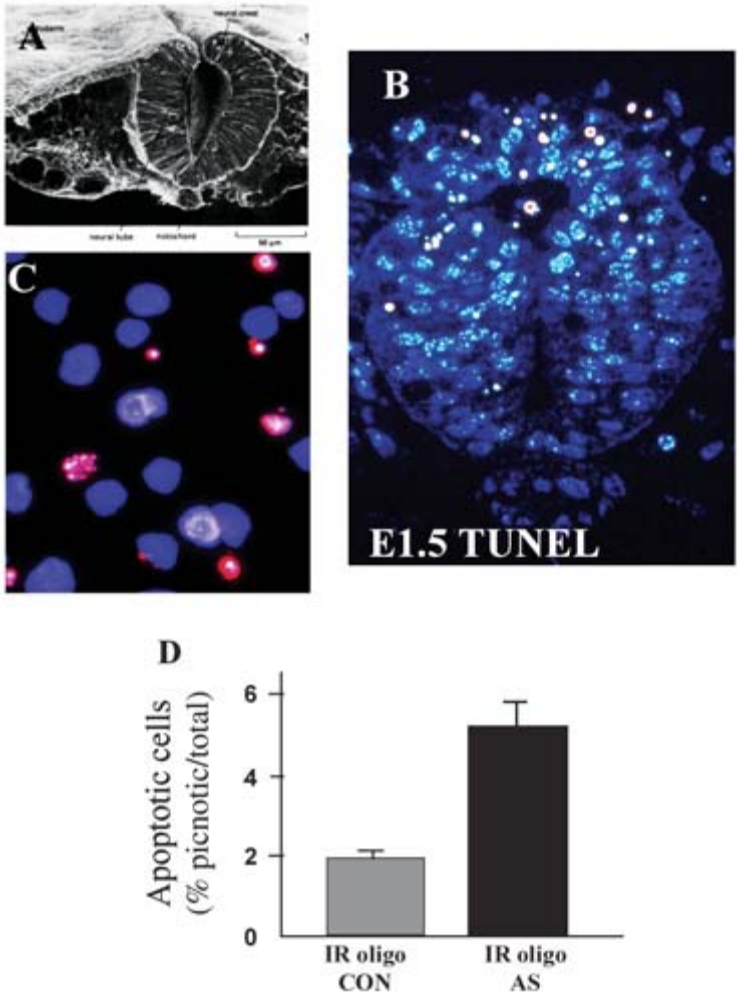

FIGURE 4. *Apoptotic cell death in the chick embryo dorsal neural tube. A) Cross-section of the neural tube by scanning electron microscopy of a E1.5 embryo. B) TUNEL assay detecting apoptotic cells, predominant in the dorsal part (upper) of the neural tube. C) Dissociated cells from the embryo stained by TUNEL (note bright apoptotic bodies). D) Mean values of percent of apoptotic cells in E1.5 embryos treated with antisense oligonucleotides against the insulin receptor (IR oligo AS) or a control (IR oligo CON).*

TABLE I. *Developmental abnormalities induced by proinsulin excess in E1,5 chick embryos*

	Nº of embryos
Control embryos abnormal	0 /13
Proinsulin treated embryos abnormal	12/15
Abnormalities:	
Asymmetric anterior neuropore closure	5/15
Flattened optic vesicles	8/15
Asymmetric rhombomers	6/15
Neural tube flexures	9/15
Reduction of apoptosis in proinsulin treated embryos:	5/5

From Hernández-Sánchez et al. 2003

IGF-I is essential for differentiation of neural stem/precursor cells in mice

After the progress made in understanding an early developmental role of proinsulin, it did not escape our attention that most investigators in the field of growth and development had analysed the possible roles of two other proteins of the family, IGF-I and II, in much more depth. Experiments using gene targeting deletion approaches generated mice lacking expression of IGF-I and II, and their receptors, alone or in combination, over a decade ago. Together with the confirmation of the essential role of each of these two factors in prenatal growth -in addition to IGF-I's role in postnatal growth, which has led to its use in human therapy (35) emerged evidence of a variety of subtle phenotypes in different tissues (3, 23, 27). From our previous studies in chick embryos we knew that IGF-I was expressed earlier during organogenesis in the nervous system than in other tissues; the availability of IGF-I knockout mice convinced us to switch to this model for a detailed analysis of the role of IGF-I in neurogenesis.

To set out a suitable system for manipulation we established highly proliferative cultures with the self-renewal characteristics of stem cells (39), obtained from the olfactory bulb of embryonic E12.5

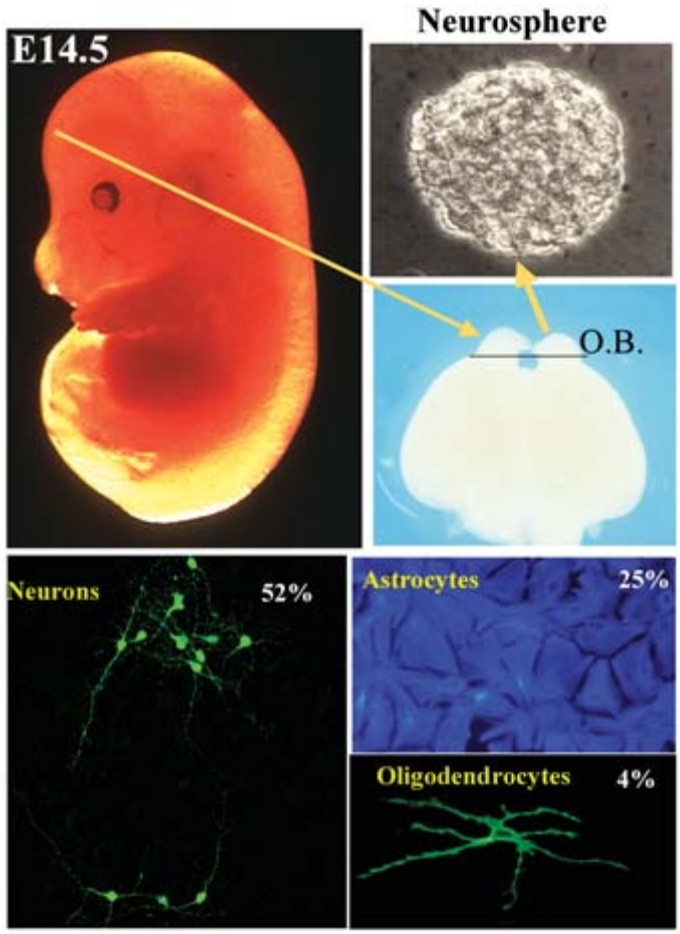

FIGURE 5. *Isolation of neural stem cells from E15 mouse olfactory bulb (O.B.). Mouse embryo O.B. are dissected from brain (line over the dorsal view indicates the section plane) and dissociated cells are placed in culture in the presence of FGF-2 and EGF plus insulin or IGF-I. The cells divide actively and after 3-4 days they form round aggregates, denominated neurospheres. After multiple passages the cells maintain their proliferative nature. When the mitogens are withdrawn (in the presence of insulin or IGF-I) and cells are plated at high density, they enter a differentiation program and, after 3-5 days, cells with the characteristics of neurons (TUJ1$^+$), astrocytes (GFAP$^+$) and oligodendrocytes (O4$^+$) can be identified in the culture. The percentages indicated are the average values obtained in a typical culture (40).*

to E14.5 mice (40) (Fig. 5). These cells, growing as neurospheres, were stimulated to proliferate by exogenous IGF-I in a dose-

dependent manner. Interestingly, insulin or proinsulin had no effect on proliferation, and only a small cell survival effect if added alone, but they were synergistic with EGF and FGF2 when added together with these two mitogens. When high insulin or low IGF-I was present and the mitogens were withdrawn, the stem cells stopped proliferating and underwent differentiation into the three types of cells characteristic of the central nervous system: neurons, astrocytes and oligodendrocytes (Fig. 5). Compared to other types of embryo-derived neural stem cells reported in the literature, the isolated olfactory bulb stem/precursor cells showed a remarkable capacity to generate neurons (52% of the differentiated cells after 3-4 days) (Fig. 5).

If instead of preparing neurospheres from olfactory bulbs of wild type mice, we used IGF-I knockout mice, the cells were able to proliferate in culture normally, whereas their ability to differentiate into neurons and glia were impaired (40). This finding agreed with in vivo data, since olfactory bulb mitral neurons were decreased in the IGF-I knockout embryos at E18.5 (32).

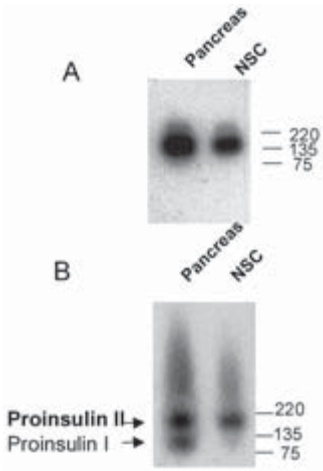

FIGURE 6. *Expression of proinsulin II mRNA in the O.B. stem cells. A) RT-PCR of a common region of proinsulin mRNA I and II followed by Southern blot with a nested oligonucleotide in pancreas and proliferative neural stem cells (NSC). B) Restriction enzyme digestion with MspI of the PCR products generated in A, followed by Southern blot shows the presence of proinsulin II in NSC.*

The fact that the cells from mice lacking IGF-I managed to maintain normal proliferation in culture suggested that perhaps they were overproducing other proteins of the insulin family or they were upregulating the IGF-I receptor to compensate. We set out RT-PCR analysis of the proinsulin I and proinsulin II mRNAs from wild type mice in a way that we could determine if one or both of the genes were expressed. In the mouse pancreas, the two mRNAs are co-expressed, whereas in the proliferating neural stem cells, only proinsulin II mRNA was found (Fig. 6). Additional experiments compared the wild type neural stem cells with those from IGF-I knockout mice and the results were variable (Fig. 7). We would need to analyze a larger number of mice to be certain that there was overcompensation at the level of proinsulin II expression in the absence of IGF-I. Even with similar amounts of proinsulin mRNA, however, different transcripts with distinct translational capacity might be present, an aspect that remains to be elucidated. More

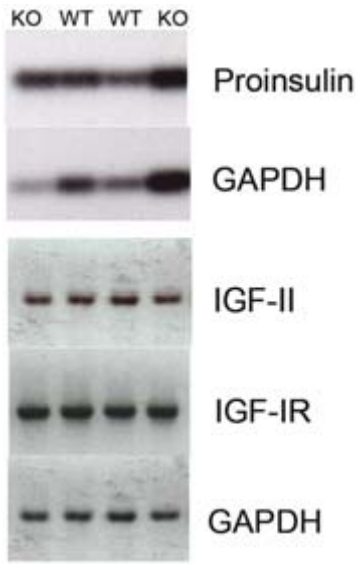

FIGURE 7. *Expression of proinsulin, IGF-II and IGF-I receptor in O.B. stem cells from wild type (WT) and IGF-I knockout mice (KO). RNA from the proliferative O.B. stem cells derived from normal mice or mice null for IGF-I was subjected to RT-PCR followed by Southern blot for the indicated genes, proinsulin, IGF-II, the IGF-I receptor and the control GAPDH (corresponding in each case to the sample(s) above.*

consistent results were observed with IGF-II and the IGF-I receptor mRNAs, neither of which appeared to change in proliferating neural stem cells under proliferative conditions, whether or not they had the IGF-I gene knockout (Fig. 7). Nevertheless, this finding does not exclude the possibility that IGF-II or the IGF-I receptor could be up-regulated at the posttranscriptional level.

In neurogenesis, therefore, the presence of proinsulin as well as IGF-I and II mRNAs suggests that all three proteins may play a role but the most clear effect observed, thus far, is the essential action of IGF-I in neural differentiation, at least in the mouse olfactory bulb.

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REFERENCES

(1) BASSAS, L.; DE PABLO, F.; LESNIAK, M. A. and ROTH, J. (1985) Ontogeny of receptors for insulin-like peptides in chick embryo tissues: early dominance of insulin-like growth factor over insulin receptors in brain. *Endocrinology* 117: 2321-2329.

(2) BANTING, F. G. and BEST, C. H. (1922): *J. Lab Clin. Med.* 7: 256-271.

(3) BAKER, J.; LIU, J. P.; ROBERTSON, E. J. and EFSTRATIADIS, A. (1993): Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*. 75: 73-82.

(4) BENECKE, H.; FLIER, J. S. and MOLLER, D. E. (1992): Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. *J. Clin. Invest* 89: 2066-2070.

(5) BEVAN, P. (2001): Insulin signalling. *J. Cell Sci.* 114: 1429-1430.

(6) CHAN, S. J. and STEINER, D. F. (2000): *Amer. Zool.* 40: 213-222.
(7) CHAN, S. J.; CAO, Q. P. and STEINER, D. F. (1990): Evolution of the insulin superfamily: cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. *Proc. Natl. Acad. Sci. U.S.A.* 87: 9319-9323.
(8) CHERVITZ, S. A. and cols. (1998): Comparison of the complete protein sets of worm and yeast: orthology and divergence. *Science* 282: 2022-2028.
(9) DE LA ROSA, E. J.; BONDY, C. A.; HERNÁNDEZ-SÁNCHEZ, C. ; WU, X. ; ZHOU, J.; LÓPEZ-CARRANZA, A.; SCAVO, L.M. and DE PABLO, F. (1994): Insulin and insulin-like growth factor system components gene expression in the chicken retina from early neurogenesis until late development and their effect on neuroepithelial cells. *Eur. J. Neurosci.* 6: 1801-1810.
(10) DE LA ROSA, E. J.; VEGA-NÚÑEZ, E.; MORALES, A. V.; SERNA, J.; RUBIO, E. and DE PABLO, F. (1998): Modulation of the chaperone heat shock cognate 70 by embryonic (pro)insulin correlates with prevention of apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 95: 9950-9955.
(11) DE PABLO, F.; ROTH, J.; HERNÁNDEZ, E. and PRUSS, R. M. (1982): Insulin is present in chicken eggs and early chick embryos. *Endocrinology* 111: 1909-1916.
(12) DE PABLO, F.; GIRBAU, M.; GÓMEZ, J. A.; HERNÁNDEZ, E. and ROTH, J. (1985a): Insulin antibodies retard and insulin accelerates growth and differentiation in early embryos. *Diabetes* 34: 1063-1067.
(13) DE PABLO, F.; HERNÁNDEZ, E.; COLLIA, F. and GÓMEZ, J. A. (1985b): Untoward effects of pharmacological doses of insulin in early chick embryos: through which receptor are they mediated? *Diabetologia* 28: 308-313.
(14) DE PABLO, F. and ROTH, J. (1990): Endocrinization of the early embryo: an emerging role for hormones and hormone-like factors. *Trends Biochem. Sci.* 15: 339-342.
(15) DUPONT, J. and LE ROITH, D. (2001): *Horm. Res.* 55 Suppl 2: 22-6, 22-26.
(16) FISHER, T. and WHITE, M. (2004): Signaling pathways: the benefits of good communication. *Curr. Biol.* 14: 1005-1007.
(17) GARCÍA DE LACOBA, M.; ALARCÓN, C., DE LA ROSA, E. J. and DE PABLO, F. (1999): Insulin/insulin-like growth factor-I hybrid receptors with high affinity for insulin are developmentally regulated during neurogenesis. *Endocrinology* 140: 233-243.
(18) GIRBAU, M.; BASSAS, L.; ALEMANY, J. and DE PABLO, F. (1989): *In situ* autoradiography and ligand-dependent tyrosine kinase activity reveal insulin receptors and insulin-like growth factor I receptors in prepancreatic chicken embryos. *Proc. Nat. Acad. Sci. U.S.A.* 86: 5868-5872.
(19) HERNÁNDEZ-SÁNCHEZ, C.; RUBIO, E.; SERNA, J.; DE LA ROSA, E. J. and DE PABLO, F. (2002): Unprocessed proinsulin promotes cell survival during neurulation in the chick embryo. *Diabetes* 51: 770-777.
(20) JONES, J. I. and CLEMMONS, D. R. (1995): Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* 16: 3-34.
(21) KASIK, J. W.; LU, C. and MENON, R. K. (2000): The expanding insulin family: structural, genomic, and functional considerations. *Pediatr Diabetes* 1: 169-177.

(22) KIMURA, K. D.; TISSENBAUM, H. A.; LIU, Y. and RUVKING, G. (1997): daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science* 277: 942-946.
(23) KITAMURA, T.; KAHN, C. R. and ACCILI, D. (2003): Insulin receptor knockout mice. *Annu Rev Physiol*. 65: 313-32.
(24) LAWLOR, M. A. and ALESSI, D. R. (2001): PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* 114: 2903-2910.
(25) MORALES, A. V.; SERNA, J.; ALARCÓN, C.; DE LA ROSA, E. J. and DE PABLO, F. (1997): Role of prepancreatic (pro)insulin and the insulin receptor in prevention of embryonic apoptosis. *Endocrinology* 138: 3967-3975.
(26) LIN, X.; TAGUCHI, A., PARK, S., KUSHNER, J. A., LI, F., LI, Y. and WHITE, M. F. (2004): Dysregulation of insulin receptor substrate 2 in beta cells and brain causes obesity and diabetes. *J. Clin. Invest.* 114: 908-916.
(27) LIU, J. P., BAKER, J.; PERKINS, A. S.; ROBERTSON, E. J. and EFSTRATIADIS, A. (1993): Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75: 59-72.
(28) MANSILLA, A.; LÓPEZ-SÁNCHEZ, C.; DE LA ROSA, E. J.; GARCÍA-MARTÍNEZ, V.; MARTÍNEZ-SALAS, E.; DE PABLO, F. and HERNÁNDEZ-SÁNCHEZ, C. (2005): *EMBO Reports* 6: 1182-1187.
(29) PANDINI, G.; FRASCA, F.; MINEO, R.; SCIACCA, L.; VIGNERI, R. and BELFIORE, A. (2002): Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J. Biol. Chem.* 277: 39684-39695.
(30) PASHMFOROUSH, M.; CHAN, S. J. and STEINER, D. F. (1996): Structure and expression of the insulin-like peptide receptor from amphioxus. *Mol. Endocrinol.* 10: 857-866.
(31) PÉREZ-VILLAMIL, B.; DE LA ROSA, E. J.; MORALES, A. V. and DE PABLO, F. (1994): Developmentally regulated expression of the preproinsulin gene in the chicken embryo during gastrulation and neurulation. *Endocrinology* 135: 2342-2350.
(32) PICHEL, J. G.; FERNÁNDEZ-MORENO, C.; VICARIO-ABEJÓN, C.; TESTILLANO, P. S.; PATTERSON, P. H. and DE PABLO, F. (2003): Developmental cooperation of leukemia inhibitory factor and insulin-like growth factor I in mice is tissue-specific and essential for lung maturation involving the transcription factors Sp3 and TTF-1. *Mech Develop.* 120: 349-361.
(33) POTTER, C. J.; HUANG, H. and XU, T. (2001): Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* 105: 357-368.
(34) SALTIEL, A. R. and KAHN, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414: 799-806.
(35) SAVAGE, M. O.; BLAIR, J. C.; JORGE, A. J.; STREET, M. E.; RANKE, M. B. and CAMACHO-HUBNER, C. (2005): IGFs and IGFBPs in GH insensitivity. *Endocr Dev.* 9: 100-106.
(36) SERRANO, J.; BEVINS, C. L.; YOUNG, S. W. and DE PABLO, F. (1989): Insulin gene expression in chicken ontogeny: pancreatic, extrapancreatic, and prepancreatic. *Dev. Biol.* 132: 410-418.

(37) SERRANO, J.; SHUDINER, A. R.; ROBERTS, C. T.; LEROITH, D. and DE PABLO, F. (1990): The insulin-like growth factor I (IGF-I) gene is expressed in chick embryos during early organogenesis. *Endocrinology* 127: 1547-1549.
(38) STARLING, E. H. (1905): *The Lancet* 2: 339-341.
(39) VICARIO-ABEJÓN, C.; COLLIN, C.; TSOULFAS, P. and MCKAY, R. D. G. (2000): Hippocampal stem cells differentiate into excitatory and inhibitory neurons. *Eur. J. Neurosci* 12: 667-688.
(40) VICARIO-ABEJÓN, C.; YUSTA-BOYO, M. J.; FERNÁNDEZ-MORENO, C. and DE PABLO, F. (2003): Locally born olfactory bulb stem cells proliferate in response to insulin-related factors and require endogenous insulin-like growth factor-I for differentiation into neurons and glia. *J. Neurosci.* 23: 895-906.
(41) WICKSTEED, B.; ALARCÓN, C.; BRIAUD, I.; LINGOHR, M. K. and RHODES, C. J. (2003): Glucose-induced translational control of proinsulin biosynthesis is proportional to preproinsulin mRNA levels in islet beta-cells but not regulated via a positive feedback of secreted insulin. *J. Biol Chem.* 278: 42080-90.
(42) HERNÁNDEZ-SÁNCHEZ, C.; MANSILLA, A.; DE LA ROSA, E. J.; POLLERBERG, G. E.; MARTÍNEZ-SALAS, E.; DE PABLO, F. (2003): Upstream AUGs in embryonic proinsulin mRNA control its low translation level. *EMBO J.* 22 (20): 5582-92.
(43) DE LA ROSA E. J., DE PABLO F. (2000)**:** Cell death in early neural development: beyond the neurotrophic theory. *Trends Neurosci.* 23 (10): 454-8. Review.