Cx36 is dynamically expressed during early development of mouse brain and nervous system

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Connexins are structural proteins that are part of the gap junctional channels which couple cells in different tissues. Connexin36 (Cx36) is a new member of the connexin gene family, found to be expressed essentially if not exclusively in neuronal cells in adult CNS of mouse, rat and man. Here we have studied Cx36 expression during murine embryonic development. Cx36 shows a highly dynamic pattern of expression. It is first (E9.5) evident in the forebrain and later its expression expand caudally in the midbrain. At E12.5 its expression correlates with major morphogenetic boundaries in the developing mouse brain, specifically with the dorsoventral telencephalic boundary and the Zona Limitans Intrathalamica. Starting at midgestation (E12.5), it is also expressed in both sympathetic and spinal ganglia, and in two longitudinal stripes along the spinal cord. *NeuroReport* 11:3823–3828 © 2000 Lippincott Williams & Wilkins.

Key words: Boundary; Connexin; Forebrain; Gap junction; Intercellular communication; Mouse embryogenesis; Spinal ganglia; ZLI

INTRODUCTION

Multicellular organisms require the coordinated response of group of cells to environmental stimuli. Intercellular channels present in gap junctions (GJs) provide a simple method of synchronizing response through the direct exchange of ions, metabolites and other messenger molecules between adjacent cells. These signaling pathways permit coordinated activities in rapid physiologic processes, such as the transmission of signals at electrical synapses [1,2], and in slower processes, such as cell growth and development [3].

GJs have been assumed to play a key role in neuromeric subdivision in the vertebrate neural tube [4], specifically in intercellular communication within the neuroepithelium [5]. In insects, segmental boundaries form barriers that isolate adjoining embryonic compartments [6]. GJs participate in the early patterning of the amphibian neural tube [7] and it has been proposed that they may be responsible for the intercellular diffusion of morphogenetic molecules to one compartment [4]. Another possibility is that GJs are modulated, either in number or state, at newly formed boundaries, thus redefining pre-existent morphogenetic fields, and perhaps contributing to position-dependent fate specification. Thus, the analysis of topographic distribution of the neuronal gap junction in the neuroepithelium bears on the related mechanism of clonal restriction. The findings waited from the analysis, when compared to the stages of developing brain, could show a relationship between them.

Gap junction channels are composed by two hemichannels, named connexons, each formed by six structural units called connexins (Cx). Each connexin protein forms gap junction that shows different electrophysiological properties, channel permeability and phosphorylation. Recently a new member of the connexin gene family has been cloned and named Cx36 [8–10]. This Cx has been localized mainly in neuronal cells, unlike the other Cxs that are mainly localised in glial cells. For example Cx43 was reported in astrocytes and some neurons [11–13], Cx32 in oligodendrocytes and some neurons [14] and Cx45 in oligodendrocytes [15]. Analysis of Cx36 mRNA distribution in adult animals revealed its intense expression in different brain regions and in the retina [8,9] with a precise topographic pattern [16].

In the present study, time of appearance, time-related changes and topographic distribution of the Cx36 during a time period ranging from blastula (E3,5) to midgestation were studied by using both radioactive and digoxigenin *in situ* hybridization on mouse embryos as well as by RT-PCR and Northern blotting, on total RNA.

MATERIALS AND METHODS

Embryos: Outbred CD-1 mice (Charles River) were mated between 21.00 h and 10.00 h. Day 0.5 pc was assumed to begin at the middle of the day of vaginal plugging. To enable a comparison of data collected by *in situ* and the Northern blotting, for each time interval, embryos used in the two experiments were taken from a single pregnant mice.

RT-PCR analysis: Total RNA from mouse brain of the developmental stages analyzed were extracted using TRI-

zol according to the manufacturer (Gibco-BRL). Five μg of total RNA extracted from E7.5, E8.5 and E9.5 mouse embryos were reverse transcribed with 150 ng random examers and 200 U RNase H^- reverse transcriptase (Superscript II Gibco BRL, Life Tecnologies, Inchinnan, UK) in 20 μl final volume reaction mixture containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 500 μM dNTP mix, 0.01 M DTT. Samples were incubated at 42°C for 50 min. Two units of RNase H were added and the incubation was continued at 37°C for 20 min. To eliminate any possibility of genomic DNA contamination, a control PCR amplification reaction was carried out on each sample of the RNA extraction. To check for any external contamination, PCR amplification was also carried out on a reaction mix sample in the absence of cDNA. PCR analysis was performed in a final volume of 25 μl, using 2.5 μl cDNA solution in a mix containing 10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 2 U REDTAQ DNA polymerase (Sigma-Aldrich, Inc), 0.2 mM dNTP mix, 20 pM of both sense (FO1: 5′-GCTCTAGACCATGGGGGAATGG ACCATCTT-3′) and antisense (RO1: 5′-CAGCTGCAGG CTCACACATAGGCAGAGTCAC-3′) Cx36 specific primers. After 3 min denaturation to activate the polymerase, 35 cycles of amplifications with Perkin-Elmer 9700 thermocycler were performed under the following conditions: melting at 95°C for 45 s; annealing at 58°C for 1 min; extension at 72°C for 42 s. The same PCR conditions were used to amplified GAPDH product from E7.5, E8.5 and E9.5 using the following sense and antisense specific primers. Amplification products were separated by agarose gel electrophoresis, recovered from gel by glass-milk method (Qiagen) and subcloned in the plasmid vector pCR-II-TOPO (INVITROGEN). DNA sequencing was performed by using thermosequenase (Amersharn).

Northern blot: Northern blot analysis of Cx36 mRNA was performed as described previously [9] using DNA probe generated from a *Xho*II/*Sac*I genomic fragment corresponding to the second exon of the mouse Cx36 gene [10]. The probe was labeled with ^{32}P using a nick translation kit (Gibco-BRL) according to the manufacturer's protocol.

The amount of total RNA on the Northern blot was standardized by hybridization to a probe of glyceraldeydephosphate dehydrogenase (GAPDH) prepared by RT-PCR amplification of a specific cDNA fragment. The densiometric analysis was carried out using a Fuji Bas analyzer.

In situ *hybridization:* *In situ* hybridization analysis was performed on both whole mount embryos and on serial sagittal, coronal and transverse paraffin sections (7 μm), treated respectively with digoxigenin and autoradiographic procedures. The Cx36 sense and antisense RNA riboprobes were labelled by incorporation of [^{35}S-α]UTP or digoxigenin-11-UTP. The probes were generated from a genomic fragment corresponding to intracellular loop of the mouse Cx36 gene [10]. The procedures for the *in situ* hybridization on sections and whole embryos were performed as previously described [17,18].

RESULTS

Total RNA extracted from blastula (E3.5) was a gift of V. Nigro (Naples). Total RNA was extracted from microdissected embryonic (E7.5, E10.5, E11.5, 13.5, E15.5, E18.5), neonatal (P0) and adult brain (P28). At stages E3.5, E7.5, E8.5 and E9.5 the mRNA levels were measured using RT-PCR (Fig. 1a). At stages E11.5, E13.5, E15.5, E18.5, P0 and P28, the mRNA levels were sampled by Northern blotting (Fig. 1b). Radioactive *in situ* hybridization was performed on sagittal sections at E7.5 (including decidual annexes) and E13.5 and on transverse sections at E12.5 and E13.5. Non-radioactive *in situ* hybridization was performed on whole mount embryos at E8.5, E9.5, E10.5, E11.5 and E13.5.

The expression of the Cx36 mRNA at lower intensity for *in situ* hybridization was identified by RT-PCR. While no RT-PCR product was found at stage E3.5 (blastocist), a very weak band was visualized at E7.5 (Fig. 1a). The intensity of this band increased slightly at E8.5 and strongly at E9.5, when *in situ* hybridization showed the first signal.

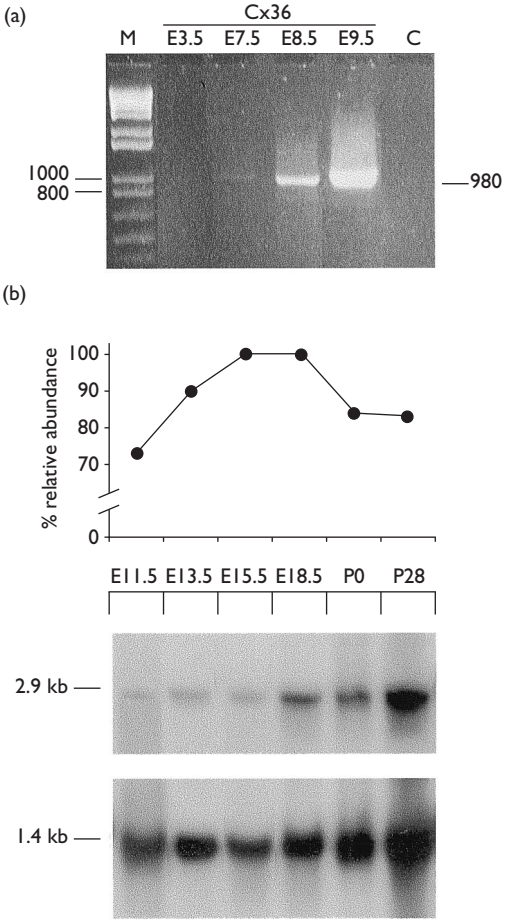

Fig. 1. (**a**) RT-PCR using oligos (FOI and ROI) on early embryonic stages shows the appearance of Cx36 at E7.5. C = PCR negative control. Controls PCR on RNA not retrotranscribed were negative for all stages analysed (data not shown). (**b**) Northern Blot analysis of Cx36 in total RNA from dissected mouse brain at stages E11.5, E13.5, E15.5, E18.5, P0 and P28. A single 2.9 kb transcript is detected hybridizing with a specific Cx36 probe. Hybridization with GAPDH (1.4 kb) has been used for normalization. The graphic shows the relative amount of Cx36 mRNA at the different stages analysed, after normalization against GAPDH. The highest level (E15.5–E18.5) has been arbitrarily indicated as 100%.

The later time course of the Cx36 mRNA was shown by Northern blotting at different stages from E11.5 to P28. A single band of 2.9 kb was found at all ages analyzed (Fig. 1b). A densitometric evaluation (Fig. 1b) indicates that Cx36 mRNA progressively increases from E11.5 to E13.5, reaching the highest level at E15.5 and E18.5. It is then reduced by about 20% at P0. At P28 the level of mRNA expression was similar to P0.

In situ hybridization on embryos was first performed on sections at E7.5. At that time no signal was detected in the brain whereas a weak signal was found in the extraembryonic decidual annexes (data not shown). At E8.5 signal was absent from the embryonic brain, while at E9.5 labeling of medium intensity was visualized in the most anterior territory of the brain. The posterior border of this territory corresponds to the area where the zona limitans intrathalamica (ZLI) will form. The signal appears to be homogeneous throughout the marked region. The expression pattern is continuous including the optic stalk and the forming optic cup (Fig. 2a,b). This pattern of expression is maintained up to E10.5 (Fig. 2c,d) when its caudal margin extends posteriorly, in the pretectum area. At this stage the hybridization signal is confined essentially to the proliferative neuroepithelium. At E12.5 the mRNA was still expressed in the forebrain region but the expression pattern has clearly evolved (Fig. 2e–g). At this time there is a strong stripe that is localized at the boundary between the dorsal telencephalon (that will give rise to the cerebral cortex) and ventral telencephalon (that will give rise to the striatum) (white arrowhead in Fig. 2f). This stripe is clearly evident on transverse sections through the telencephalon (white arrowheads in Fig. 2f.h–i). A second stripe is present at the division between dorsal and ventral thalamus in a region that is indicated as ZLI (black arrowhead in Fig. 2f,g; white arrowhead in Fig. 2l). These two stripes coincide with the two most important boundaries in the forebrain, which play specific functions during brain morphogenesis. The Cx36 mRNA is still expressed in the dorsal telencephalon but the density is much weaker than in previous stages. Finally at the level of the isthmus two strong signals on his ventral part were detected (open arrowhead in Fig. 2e). Weak signals were detected in both sympathetic and spinal ganglia, along the trunk as well as longitudinal stripes in the spinal cord. At E13.5 the hybridization signal previously reported in the dorsoventral boundary of the telencephalon is now restricted to the most ventrolateral aspect (arrowhead in Fig. 2k). Additional expression signals were localized in both the ventral diencephalon (Fig. 2l) and in the region that will give rise to the superior colliculus, as well as in the region of the rhombic lip, that will give rise to the cerebellum (Fig. 2m,n). A strong signal was also detected in the glomerular layer of the olfactory bulb (Fig. 2o). The hybridization signal previously reported in both sympathetic (Fig. 3a,c) and spinal ganglia (Fig. 3b) became very strong. Similarly the expression of the Cx36 mRNA in the spinal cord as sagittal stripes also increased. It is worth noting that signals of the spinal cord, which remained strongly positive up to E17.5 (data not shown), may correspond to the developing motoneurons that are strongly positive in adult life (Fig. 3e–h) [16].

As shown in Table 1, at earlier stages (E9.5–E10.5), the hybridization signal is homogeneous along the proliferating neuroepithelium, while at the latest stages (E12.5–E13.5) Cx36 is expressed at different intensity in specific areas of the developing brain.

DISCUSSION

Several connexins are expressed during preimplantation stages (from cleavage to blastula stage) in the mouse (e.g. Cx31 and Cx43) [19]. Our RT-PCR has shown that Cx36 mRNA is not expressed in the mouse blastula (E3.5) and is only faintly expressed at E7.5. At this stage the Cx36 was expressed in decidual annexes, that is in extraembryonic tissue. Using whole mount *in situ* hybridization, the first signal in embryonic structure was evident only at E9.5. It was specifically localized in the most anterior region of the developing brain, that is in the forebrain. It is worth nothing that at this time the forebrain is undertaking a process of segmentation in different areas, called prosomeres [20]. Thereafter, the expression of the Cx36 was very dynamic, showing a general pattern of diffusion from rostral to caudal CNS. First expression of the Cx36 was found in the rostral forebrain, thereafter it appeared in prosomeres progressively more caudal (see Table 1). No expression was detected in prosomere 2 (see Table 1). It is known that maturation of the CNS proceeds caudal to rostral in the embryo [21]. Thus, Cx36 expression pattern does not to correlate to this maturation gradient. On the contrary it is expressed widely in the forebrain just when neurogenesis is about to start (E9.5–E10.5). Later (E12.5–E13.5) its expression moved to posterior brain and spinal cord structures. Other genes, mainly transcription factors such as BF1 and BF2 [22] or Otx1 [22] showed a similar pattern of expression.

Cx36 is the earlier expressed connexin in the rodent brain at mouse E9.5. Other connexins such as Cx43 and Cx32 appear later in development, at E12 and P14 onwards, respectively, in the rat brain [23].

The bimodal pattern of expression of Cx36 showed by the *in situ* hybridization analysis could be correlated with two major events during early embryonic CNS development. Cx36 was first expressed (E9.5–E10.5) through the rostral forebrain. Thus, during these stages the Cx36 GJs could be mainly involved in coupling of neuroepitelial cells before or at the onset of neurogenesis, possibly establishing a functional compartment enabling direct cell–cell communication. Soon after, at E12.5 its expression was mainly localized along the two most important boundaries of the forebrain, such as the ZLI and division between dorsal and ventral telencephalon. Thus, at this stage the Cx36 GJs could be involved in delimiting subcompartments or in defining properties of the boundaries themselves via direct coupling. This finding suggest a direct role of the Cx36 GJs in brain morphogenesis. In fact, Cx36 GJs may play a role in confining the intercellular diffusion of morphogenetic molecules to single compartment, in agreement with previous studies [4]. These observations lead to the question about the nature of the molecules that are confined or are able to pass through these Cx36 GJ channels located at these boundaries. Further investigations must be planned to answer to these questions.

While *in situ* hybridization analysis focused on the spatiotemporal dynamic pattern during early embryogen-

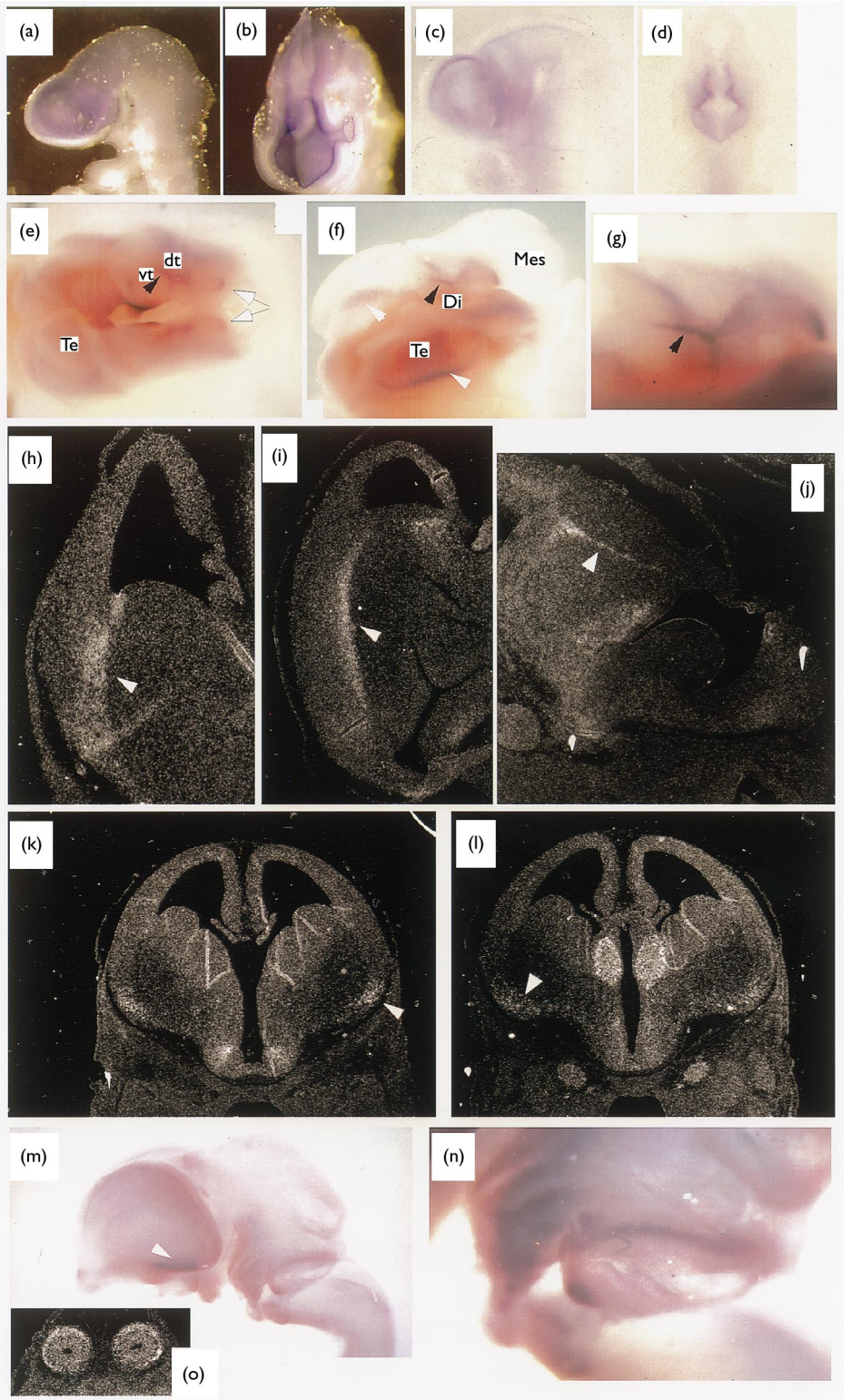

Fig. 2. mCx36 expression during early mouse embryo development in whole mount embryos at E9.5 (**a**,**b**), E10.5 (**c**,**d**) E12.5 (**e**–**g**) and whole mount brain at E13.5 (**m**,**n**), as well as in transverse (**h**,**i**) and sagittal (**j**) sections at E12.5, and transverse at E13.5 (**k**,**l**,**o**). dt, dorsal thalamus; vt, ventral thalamus; Di, diencephalon; Te, telencephaton; Mes, mesencephalon; Met, metencephalon. Black arrowheads indicate signal in the ZLI, white arrowheads indicate signal along the dorsoventral boundary in the telencephalon. An arrow in (M) indicate signal at the level of the dorsoventral boundary in the telencephalon.

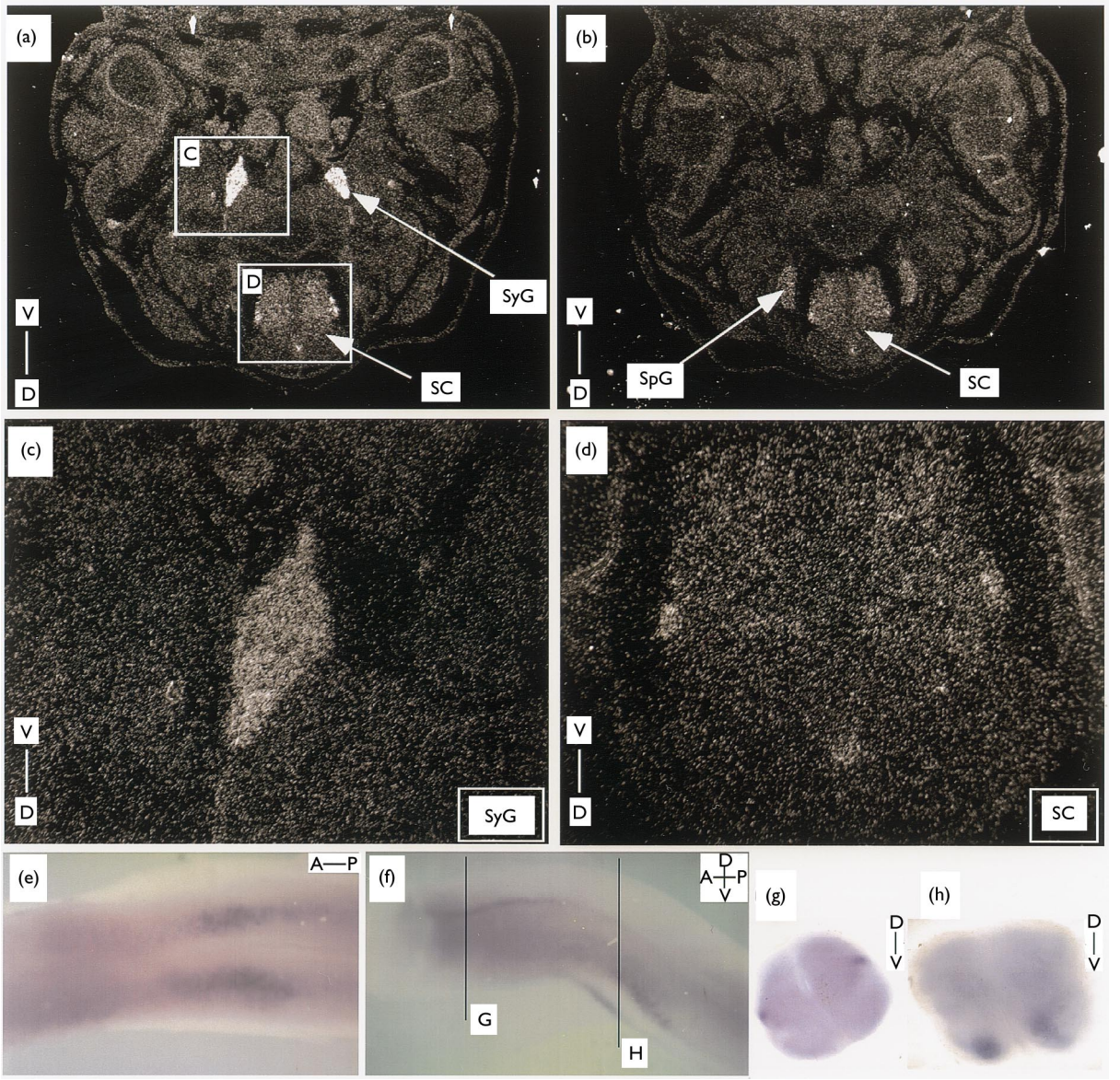

Fig. 3. mCx36 expression during spinal cord development in transverse sections (**a**–**d**) at 12.5, as well as in whole mount spinal cord at E14.5 (**e**) and E17.5 (**f**–**h**). (c) and (d) are inset in (a). Both spinal and sympathetic ganglia express Cx36. In the spinal cord two stripes of expression, one lateral and onenset in (a). Both spinal and sympathetic ganglia express Cx36. In the spinal cord two stripes of expression, one lateral and one ventral are present. A-P = anterior-posterior; D-V = dorsoventral; SC = spinal cord; SpG = spinal ganglia; SyG = sympathetic ganglia.

esis up to E13.5, Northern blot time course analysis extended up to newborn (P0) and to adult (P28), first to picture a complete curve of the expression levels during gestation and second to compare these levels to newborn and adulthood. We found a peak of expression at E15.5–E18.5 followed by a decrease at P0. This value is comparable to what found at P28. It is worth nothing that the expression of the Cx36 between these two dates was recently analyzed by Prime [24]. They found that the time-related changes in the relative abundance of the Cx36 progressively increased from P0 to a peak at about P12 and thereafter reduced to P25 at values comparable to those at P0. Taken together these findings point out that the expression of the Cx36 in developing brain has two peaks: one at about E15.5 and the other at about P12. The first peak grossly corresponds to the time of greatest neurogenesis of the cortical cells [21]. The second peak, which corresponds to the time in which the neurons of the cerebral cortex are directly coupled [25], is probably associated with the first environmental stimuli occurring just after birth.

REFERENCES

1. Bennet MVL. *J Neurocytol* **26**, 349–66 (1997).
2. Brivanlou IH, Warland DK and Meister M. *Neuron* **20**, 527–39(1998).
3. Simon AM, Goodenough DA, Li E and Paul DL. *Nature* **385**, 525–29 (1997).

Table 1. Cx36 mRNA expression in the mouse forebrain at E10–E13.5, according to the prosomeric model [20].

	E9.5	E10.5	E12.5	E13.5
Secondary prosencephalon				
Prosomere 6	++	++		
Olfactory bulb			++	+++
Lateral septum			+++	++
D/V telencephalic border		+++	+++	
Prosomere 5	++	++		
Neocortex			+	+
Lateral hypothalamus			++	++
Prosomere 4	++	++		
Archicortex			+	+
Diencephalon				
Prosomere 3	++	++		
Ventral thalamus			++	+
Zona limitans intrathalamica			+++	+++
Prosomere 2	–	–	–	–
Prosomere 1				
Pretectum			+	+

Low (+), moderate (++) and high (+++) levels of Cx36 expression are shown. Undetectable expression is also indicated (–).

4. Martinez S, Geijo E, Sanchez-Vives MV *et al. Development* **116**, 1069–1076 (1992).
5. Minkoff R, Parker SB and Hertzberg EL. *Development* **111**, 509–522 (1991).
6. Lawrence PA. *J Embryol Exp Morphol* **30**, 681–699 (1973).
7. Warner AE. *J Physiol* **235**, 267–286 (1973).
8. Condorelli DF, Parenti R, Spinella F *et al. Eur J Neurosci* **10**, 1202–1208 (1998).
9. Sohl G, Degen J, Teubner B and Willecke K. *FEBS Lett* **428**, 27–31 (1998).
10. Cicirata F, Parenti R, Spinella F *et al. Gene* **27**, 123–130 (2000).
11. Dermietzel R, Traub O, Hwang TK *et al. Proc Natl Acad Sci USA* **86**, 10148–10152 (1989).
12. Giawne C, Fromaget C, el-Aoumari A *et al. Neuron* **6**, 133–143 (1991).
13. Simburger E, Stang A, Kremer M and Derinietzel R. *Histochem Cell Biol* **107**, 127–137 (1997).
14. Nadarajah B, Thomaidou D, Evans WH and Parnavelas JG. *J Comp Neurol* **376**, 326–342 (1996).
15. Kunzelmann P, Blumcke I, Traub O *et al. J Neurocytol* **26**, 17–22 (1997).
16. Parenti R, Gulisano M, Zappalà A and Cicirata F. *Neuroreport* **11**, 1497–1502 (2000).
17. Gulisano M, Broccoli V, Pardini C and Boncinelli E. *Eur J Neurosci* **8**, 1037–50 (1996).
18. Hogan B, Beddington R, Costantini F and Lacy E. *A Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1994.
19. Davies TC, Barr KJ and Jones DH. *Dev Genet* **18**, 234–243 (1996).
20. Puelles L and Rubenstein JLR. *Trends Neurosci* **16**, 472–479 (1993).
21. Bayer SA and Altman J. *Neocortical Development*. New York. Raven Press (1991).
22. Rubenstein JL, Shimamura K, Martinez S and Puelles L. *Annu Rev Neurosci* **21**, 445–477 (1998).
23. Nagy JI and Rash JE. *Brain Res Brain Res Rev* **32**, 29–44 (2000).
24. Prime G, Horn G and Bend S. *Dev Brain Res* **119**, 111–115 (2000).
25. Yuste R, Nelson DA, Rubin WW and Katz LC. *Neuron* **14**, 7–17 (1995).

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