Expression pattern of the maternally imprinted gene Gtl2 in the forebrain during embryonic development and adulthood

D. McLaughlin [a,b,1,2], M. Vidaki [a,b,1], E. Renieri [a], D. Karagogeos [a,b,*]

[a] *Department of Basic Science, University of Crete Medical School, 71110 Heraklion, Greece*
[b] *Institute of Molecular Biology and Biotechnology (IMBB), 71110 Heraklion, Greece*

Abstract

Recent work has uncovered a large number of imprinted genes, many of which are thought to play a role in neurodevelopment and behavior. In order to begin to understand the role of specific genes in these processes, their expression patterns will be key. In this study we used in situ hybridization to study the developmental expression of *Gtl2* in the forebrain from E12.5 to adulthood, since preliminary data from a microarray study indicated differential expression between the ventral and dorsal telencephalon of the mouse at a critical time point in the generation and migration of cortical neuronal populations. Strong expression was observed in the diencephalon, ventral telencephalon, post mitotic cell layers of the neocortex and pyramidal cell layer of the hippocampus. Additionally, heavily labeled subpopulations of laminar restricted cells were seen in the latter two areas.
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1. Results and discussion

The gene trap locus 2 (Gtl2) was identified in a gene trap screen to identify developmentally important genes (Schuster-Gossler et al., 1994). Gtl2 (or maternally expressed gene3, Meg3) is expressed from mouse distal chromosome 12, one of at least 10 imprinted regions in the mouse (Peters and Beechey, 2004). Gtl2[Lacz] mice which have an insertional mutation on chromosome 12 have been found to have growth retardation when the transgene is inherited from their father (Schuster-Gossler et al., 1996). Some imprinted genes, and all those which are expressed from the maternally inherited chromosome are thought to act as microRNAs (or miRNAs), noncoding RNAs of 21–25 nucleotides, which may act by repressing translation or by RNA interference (RNAi) (Seitz et al., 2003, 2004). Approximately 70 imprinted genes have been currently identified, a large number of them very recently. Imprinted

genes are genes which are expressed only from the parental allele (Reik and Walter, 2001); many of them are expressed in the brain (Davies et al., 2005). Imprinted genes are known to be involved in growth and development (Reik and Walter, 2001) and are increasingly thought to play a role in neurodevelopment and behavior (Davies et al., 2005).

The expression of *Gtl2* has been reported in the yolk sac, paraxial mesoderm, epithelial ducts of developing excretory organs and in the central nervous system by Northern blotting and in situ hybridization (Schuster-Gossler et al., 1998), though the detailed expression pattern in the brain has not been reported. The main aim of the present work was to map at high resolution the expression pattern of *Gtl2* in the forebrain using in situ hybridization, since preliminary data from our lab identified *Gtl2* as differentially expressed between the ventral and dorsal telencephalon by microarray analysis. The gene is turned on in the forebrain early on, at E9.5 (Schuster-Gossler et al., 1998 and data not shown). Detailed analysis of coronal sections of the forebrain at E12.5 showed strong expression in the basal forebrain at a distance from the ventricular zones (Fig. 1A). This may indicate that the cells expressing this gene are likely to be postmitotic neurons since in the telencephalon, glial cells are generated later (Bayer and Altman, 1990; Qian et al., 2000). No expression is observed in the neocortex at E12.5 though by E14.5 a continuous domain of expression is clearly seen to extend from the lateral aspect of the ventral
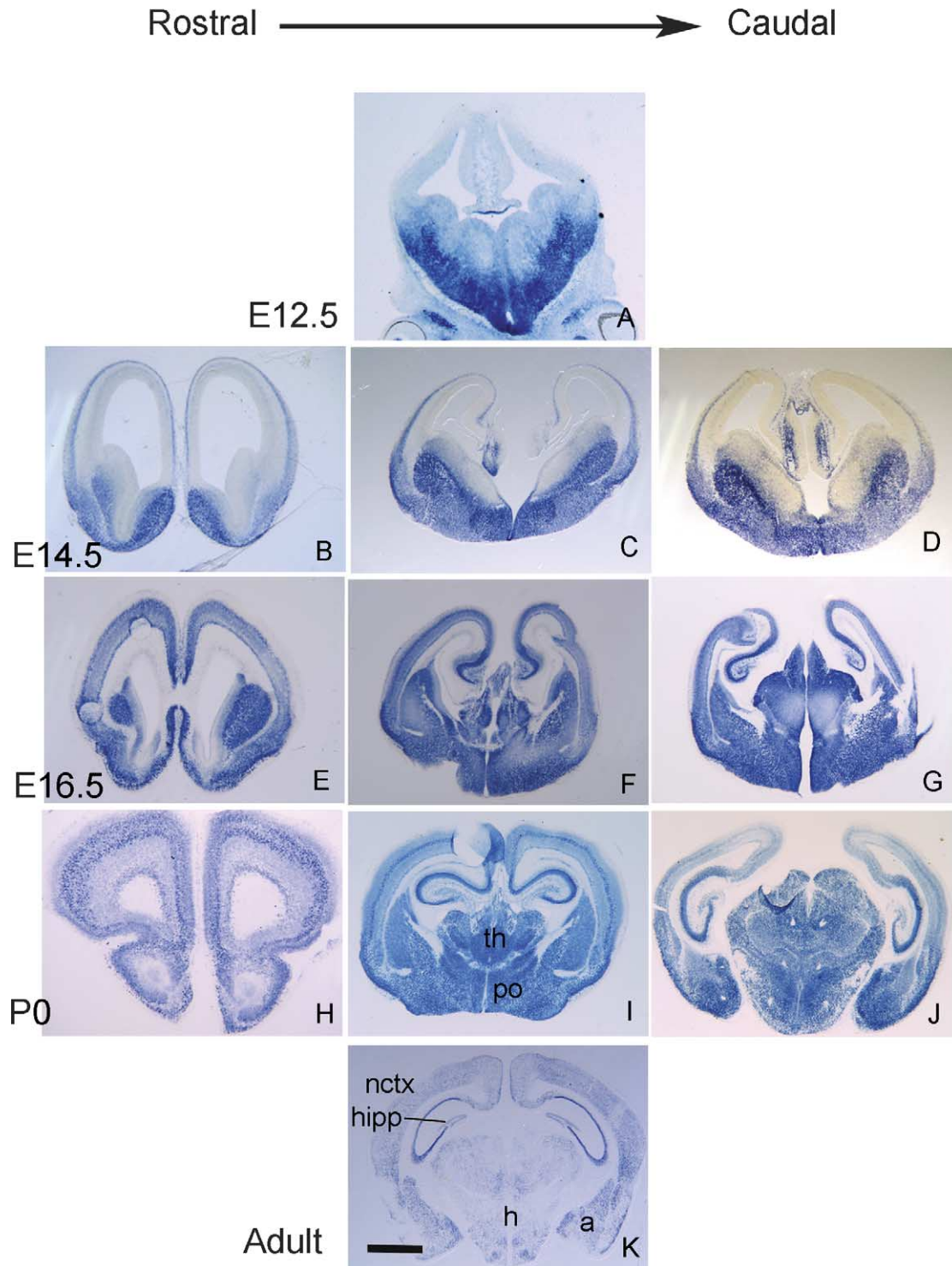
Fig. 1. Expression of *Gtl2* in the developing and adult forebrain. Coronal sections of the forebrain from E12.5 to adult showing expression of *Gtl2* at E12.5 (A), E14.5 (B–D), 16.5 (E–G) P0 (H–J) and Adult (K). For E14.5, E16.5 and P0, coronal sections are shown from rostral to caudal extents of the forebrain at comparative levels between ages. *Gtl2* expression is maintained from E12.5 to adulthood throughout spatially restricted domains in the neocortex (nctx), hippocampus (hipp), thalamus (th), hypothalamus (h), amygdala (a) and preoptic area (po). Scale bar: 300 μm.

telencephalon into the dorsal and medial telencephalon (Fig. 1B–D). This pattern of expression is maintained at E16.5 throughout the rostro-caudal length of the neocortex. Additionally, strong expression can be seen in the thalamus, dorsal thalamus, hypothalamus and hippocampus, which are rapidly developing structures at this age (Fig. 1E–G). Strong

levels of expression are maintained in these structures as well as in the olfactory bulb and amygdala at birth (Fig. 1H–J). Expression in the adult is also maintained although it appears more restricted suggesting that expression may be limited to a more specific cell type, with the exception of the hippocampus (Fig. 1K). Other regions of *Gtl2* expression include the

diencephalon (thalamus, hypothalamus), the cerebellar anlage, the medulla and the spinal cord (data not shown).

Two possibilities exist for the origin of the cells expressing *Gtl2* in the cortex. The first is that they originate in the cortex since it can be seen that *Gtl2* expression increases in relation to cortical plate expansion during development (Fig. 2). Post mitotic cells moving away from the ventricular and/or sub ventricular cortical zones may start to express *Gtl2* as they
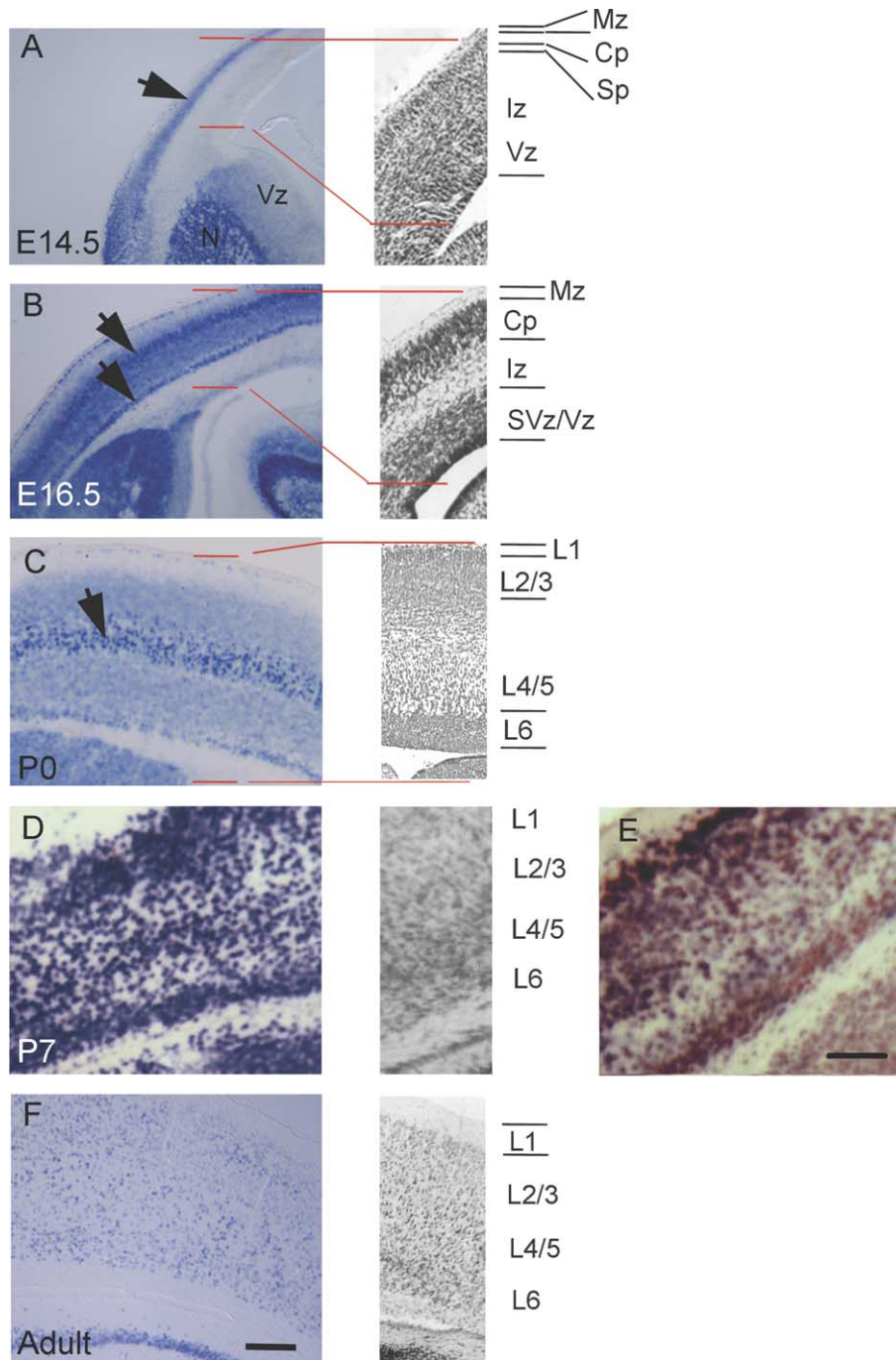

Fig. 2. Developmental regulation of *Gtl2* expression in the developing neocortex. (A–D) Coronal sections of the dorsal forebrain at E14.5, 16.5, P0, P7 and adult, respectively, show restricted expression of *Gtl2* within the neocortex to the postmitotic cell layers. Cresyl violet stained sections at comparable levels are included to indicate cortical lamination. Red coloured bars on *Gtl2* stained sections indicate the corresponding position of the cresyl violet section. (A) *Gtl2* expression at E14.5 approximates to the position of the cortical plate (arrowhead). (B) A broader domain of expression is observed at E16.5 which approximates to the intermediate zone. A band of more heavily labeled cells either side (arrowheads) may correspond to cortical cells born at different times which are migrating through the intermediate zone towards the cortical plate. (C) The domain of expression appears to extend to all areas of the cortex containing postmitotic cells at P0, apart from the outer marginal zone. Additionally, a layer more heavily labeled cells is present in layers 4/5 (arrowhead). (D) All cortical neurons express *Gtl2* at P7. (E) Immunostaining for the neuronal marker NeuN on the *Gtl2*-hybridized sections at P7. Extensive colocalization of the two signals is observed. (F) An even and sparse distribution of *Gtl2* positive cells is located throughout layers 2–6 in the adult. The signal is greatly reduced. Mz, Marginal zone; Cp, cortical plate; Iz, intermediate zone; Svz/Vz, sub ventricular and ventricular zone; L1-6, cortical layers 1–6, respectively. Scale bar: 100 μm Scale bar (D,E): 640 μm.

migrate into their positions within the cortical plate, and this may also account for the laminar differences in expression intensity observed at E16 and P0 reflecting differences in expression between cells born on different days (i.e. early in the deeper layers, later in the more superficial layers; arrowheads in Fig. 2B,C). At P7 a strong signal is observed throughout the cortical layers (Fig. 2D). It is plausible that *Gtl2* plays a role early during the development (i.e. migration) of cortical neurons up to P0, whereas at P7 it may play a role in a later event, necessary for all cortical postnatal neurons. Immunostaining of the hybridized sections with the panneuronal marker NeuN shows extensive co-localization with the *Gtl2* signal (Fig. 2E). In the adult, the level of expression is significantly reduced throughout the cortex (Fig. 2F) and is maintained in the hippocampus (Figs. 1K, 2F, 4C).

An alternative possibility is that cells expressing *Gtl2* originate as interneurons in the ganglionic eminence and continue expressing the gene during subsequent tangential migration into the cortex (see Flames and Marin, 2005 for a recent review).

In order to test the possibility that *Gtl2* expressing cortical cells are interneurons derived from the ganglionic eminence, we performed in situ hybridization in consecutive sections of telencephalic tissue with *Gtl2* and *GAD67*, an enzyme required for GABA production in interneurons as well as *Gtl2* and the transcription factor *Lhx6*, a marker of migrating interneurons from the medial ganglionic eminence which forms the largest contributor of cortical interneurons (Lavdas et al., 1999). At E14.5, a peak time point in interneuron migration to the cortex, at both rostral and caudal levels, no overlap in expression was observed (Fig. 3), thus rendering unlikely that *Gt12* is expressed by interneurons.

Strong expression of *Gtl2* was also observed in the developing hippocampus from the time of generation of this structure. A progressively restricted domain of expression was observed E16.5 to adulthood which corresponds to the increasing cellular organization at this time (Fig. 4A–C). The most intense layer of expression appeared to correspond to the pyramidal cell layers with a weaker expression throughout the dentate gyrus. Additionally, subpopulations of cells throughout this layer strongly express *Gtl2* (Fig. 4 grey arrowheads).

In summary we have analyzed in detail the expression pattern of *Gtl2* in the forebrain. *Gtl2* expression from E12.5 till postnatally is strong in the ventral telencephalon, neocortex, hippocampus and diencephalon consistent with a role in neuronal development and differentiation. Additionally, the developmental regulation of highly restricted domains of expression, both in the neocortex and hippocampus may implicate *Gtl2* in the development of specific subsets of cells within these tissues.

2. Experimental procedures

For in situ hybridisation, brains were dissected from C57/Bl6xCBA mice at E12.5, E14.5, 16.5, P0, P7 and adult, fixed in 4% PFA (or perfused in the case of postnatal and adult brains), frozen on dry ice and sectioned coronally (14 μm). All sections were stored on superfrost + slides at −80 °C, before being oven-dried at 50 °C for 20 min, subjected to proteinase treatment (10 μg Proteinase K/ml PBS) for 10 min at room temperature and fixed in cold 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 20 min. They were then washed in PBS and incubated in 0.1 M PBS + 0.1% Tween20 for 30 min. Prehybridisation was performed for 2 h at 65 °C in 50% formamide/50% 5×SSC buffer. Hybridisation was performed in humidified conditions for 16 h at 65 °C in the same buffer as for prehybridisation with DIG-labeled probe added (0.4 μg/ml) generated by in vitro transcription from plasmids containing cDNA for Gt12 (clone c10.5/c7E/H1.9, a generous gift from A. Gossler), gad67 (a gift from Dr F. Guillemot, Mill Hill, UK) and Lhx6 (Grigoriou et al., 1998). Sections were sequentially washed in 2×SSC (30 min, room temperature), 2×SSC (1 h, 65 °C), 0.2×SSC (1 h, 65 °C), PBS/0.1%
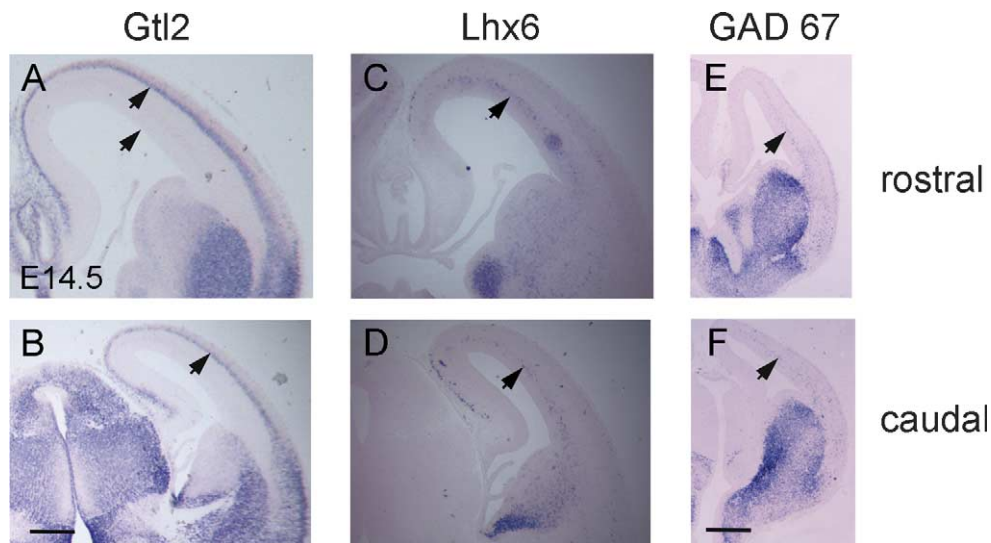

Fig. 3. Comparative expression of *Gtl2*, *Lhx6* and *GAD67* at rostral and caudal levels at E14.5. Top panels represent rostral regions and bottom panels represent caudal regions of E14.5 telencephalic tissue. Continuous expression of *Gtl2* can be observed at rostral (A) and caudal levels (B) of the forebrain extending from ventral to dorsal aspects (dark arrowheads). Expression in the cortex at both levels appears restricted to the cortical plate. Expression of *Lhx6* and *GAD67* on consecutive sections shows a similar ventral–dorsal domain of expression (arrowheads in C, D for *Lhx6* and E, F for *GAD67*), although the laminar domain of expression of *Lhx6* and *GAD67* expression appears closer to the ventricular zone than that of *Gtl2*, and approximates to the cortical intermediate zone. Scale bar, 800 μm.
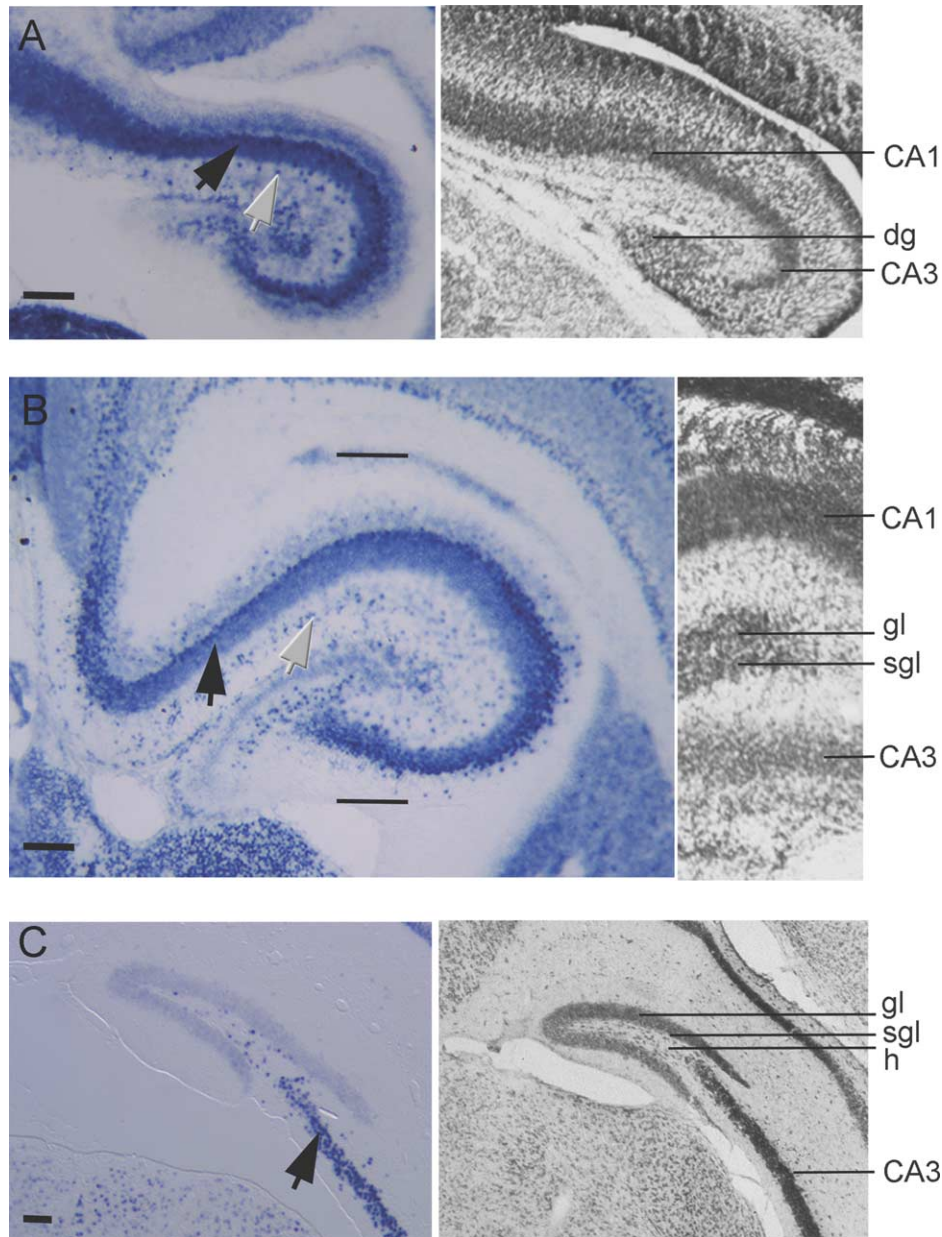
Fig. 4. Developmental regulation of *Gtl2* expression in the hippocampus. (A–C) Coronal sections from the hippocampus at E16.5, P0 and adult, respectively, show strong levels of *Gtl2* expression throughout the pyramidal cell layer of the hippocampus (arrows) and weaker staining in the dentate gyrus. Comparative cresyl violet stained sections are included showing cell density. The area of the cresyl violet section shown in B, lies between the bars in the adjacent section. At E16.5 and P0 many individually heavily labeled cells are also noted adjacent to the pyramidal cell layer (grey arrow heads). dg, dentate gyrus; gl, granular layer; sgl, sub granular layer; CA1, CA3, pyramidal cell layers; H, hylus. Scale bar: 50 μm.

Tween 20 (10 min, 65 °C) and PBS/Tween 20 (10 min, room temperature) before being treated with blocking reagent (0.1% Tween 20, 20% fetal calf serum, 5% milk powder in PBS) for 2 h at room temperature. Antibody reaction was performed by incubating the slides for 16 h at 4 °C in a 1:5000 dilution of anti-DIG alkaline phosphatase-coupled FAB fragment (Roche, Germany) in blocking solution. Sections were washed thoroughly in PBS/0.1% Tween 20 and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl pH9.5, 100 mM NaCl, 50 mM MgCl$_2$ in sterile water) for 5 min. Alkaline phosphatase activity was detected with 45 μl/ml 4-nitrobluetetrazolium chloride (NBT, Promega, USA) and 35 μl/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Promega, USA) in alkaline phosphatase buffer for ≥2 h at room temperature. The reaction was stopped with PBS. Equivalent sections were hybridised with antisense and sense probes for each gene to ensure the specificity of the hybridisation signals. In each case, there was no signal evident. Immunocytochemistry was performed as previously described (Denaxa et al., 2001). Monoclonal anti-mouse NeuN (Chemicon), goat anti mouse IgG conjugated to biotin secondary antibody (Boehringer-Mannheim) and DAB (Sigma) were used.

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