*Neuroscience* 117 (2003) 337–346

# POSTNATAL DEVELOPMENT OF THE VESICULAR GABA TRANSPORTER IN RAT CEREBRAL CORTEX

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**Abstract—Light and electron microscopic immunocytochemical techniques and Western blotting were used to investigate the postnatal development of the vesicular GABA transporter (VGAT) in the rat somatic sensory cortex.**

**VGAT immunoreactivity was low at birth, it increased gradually through the first and second weeks of life and achieved the adult pattern during the third week. At postnatal day (P)0–P5, VGAT immunoreactivity was associated exclusively to fibers and puncta. Electron microscopic studies performed at P5 showed that all identified synaptic contacts formed by VGAT-positive axonal swellings were of the symmetric type and that a substantial proportion of the boutons appeared not to have formed synapses. From P10 onward, labeled puncta were both scattered in the neuropil and in apposition to unstained cellular profiles; VGAT was also expressed in few GABAergic cell bodies. Western blottings at the same postnatal ages revealed a 55-kDa band whose intensity was weak at P0 (17% of adult), it increased constantly until P15 (P2: 35%; P5: 44%; P10: 68%; P15: 97%), and then leveled off.**

**Overall, the present results show that during neocortical development the expression of VGAT slightly precedes the complete maturation of inhibitory synaptogenesis and suggest that it may contribute to the formation of neocortical GABAergic circuitry.** 

**Key words: GABA, synaptic vesicles, neurotransmitter uptake, development, GAT-1.**

The role of GABA is prominent in the mature cerebral cortex in both physiological and pathological conditions (Cherubini and Conti, 2001) and during early development (Lauder, 1993; Ben-Ari et al., 1994; LoTurco et al., 1995; Barker et al., 1998; Belhage et al., 1998; Owens and Kriegstein, 2002; Ben-Ari, 2002).

The maturation of the neocortical GABAergic circuitry is a complex and protracted process (Owens and Kriegstein, 2002; Ben-Ari, 2002). Numerous studies have focused on the beginning of GABA-mediated responses (Cherubini et al., 1991; Luhmann and Prince, 1991; Agmon et al., 1996; Owens et al., 1999; Dammermann et al., 2000; Hanganu et al., 2001; Kilb and Luhmann, 2001), the temporal pattern of formation, maturation and pruning of inhibitory synapses (Kristt and Molliver, 1976; Blue and Parnavelas, 1983a,b; Schutz and Palm, 1989; Micheva and Beaulieu, 1995, 1996; DeFelipe et al., 1997), and the expression of synthesizing enzymes and of receptors and transporters (Lauder et al., 1986; Cobas et al., 1991; Del Rio et al., 1992; Laurie et al., 1992; Poulter et al., 1992, 1993; Fritschy et al., 1994; Vincent et al., 1995; Evans et al., 1996; Yan et al., 1997), but several details are still elusive or controversial (Varju et al., 2001; Ben-Ari, 2002; Owens and Kriegstein, 2002).

Before their release into the cleft, neurotransmitters are taken up and accumulated in synaptic vesicles by the action of integral membrane proteins named vesicular transporters (Liu and Edwards, 1997; Eiden, 2000). GABA is transferred into synaptic vesicles by a vesicular transporter termed VGAT, that is crucial for GABAergic function (McIntire et al., 1993; Rand et al., 2000; Shingai, 2000; Takamori et al., 2000) and whose activity depends on pH and electrical gradients (McIntire et al., 1997; Sagné et al., 1997). In the brain and spinal cord of adult rats, VGAT is localized to inhibitory axon terminals of both GABAergic and glycinergic neurons, where it is associated with synaptic vesicles (Chaudhry et al., 1998; Dumoulin et al., 1999). To date, nothing is known of the expression of VGAT during cortical development. In the developing neocortex, glycine (Gly) receptors are activated by an endogenous ligand other than Gly, in all likelihood taurine (Flint et al., 1998; see also Mori et al. [2002] for data on the rat hippocampus), in line with the observation that Gly levels (Aprison et al., 1969) and Gly transporter (GLYT) 1 and GLYT2 expression (Zafra et al., 1995) are very low at early postnatal ages. These findings imply that in the developing neocortex VGAT-mediated Gly transport, if any, would be negligible and thus indicate that VGAT activity would be almost exclusively associated with GABA transport. Knowledge of the developmental profile of VGAT expression could thus shed some light on the maturation of the molecular machinery involved in the establishment of cortical GABAergic circuits.

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*Abbreviations:* CP, cortical plate; DAB, 3,3' diaminobenzidine tetrahydrochloride; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; GAD, glutamic acid decarboxylase; GAT-1, plasma membrane GABA transporter 1; GAT-3, plasma membrane GABA transporter 3; Gly, glycine; GLYT, glycine transporter; IgG, immunoglobulin G; ir, immunoreactivity; NBCS, newborn calf serum; P, postnatal day; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SI, first somatic sensory cortex; TRITC, tetramethylrhodamine isothiocyanate; VGAT, vesicular GABA transporter.

0306-4522/03$30.00+0.00 
doi:10.1016/S0306-4522(02)00864-3

We therefore used immunocytochemical and Western blotting techniques to investigate the postnatal development of VGAT expression in the rat neocortex.

## EXPERIMENTAL PROCEDURES

Sprague–Dawley albino rats (Charles River, Milan, Italy; $n=95$) were used at different postnatal ages. Their care and handling were approved by the Ethical Committee for Animal Research of the University of Ancona. All efforts were made to minimize animal suffering and the number of animals used.

The age groups studied were: P0 (postnatal day 0, within 24 h of birth), P2, P5, P10, P15, P20, P30 and P60 (adult). Five animals per age group were used for immunoperoxidase studies; two rats from groups P10, P15 and P20 were used for double-labeling investigations; and three rats were used at P2 and P5 for electron microscopy. In addition, seven (at P0–P5) and five (at P10, P15, P20, P30, and P60) animals were used for immunoblotting studies. Tissue and homogenates from some of the animals included in these studies have also been used for other studies (Minelli et al., 2003a,b).

### Tissue preparation

Rats were anesthetized with 30% chloral hydrate and perfused transcardially with 0.1-M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1-M phosphate buffer (PB; pH 7.4). For electron microscopy, rats were perfused with a solution containing 3% PFA and 2% glutaraldehyde in PB. For double-labeling, rats were perfused with 4% PFA plus 0.5% glutaraldehyde in PB. Brains were postfixed for 8–12 h at 4 °C in the same fixative used for perfusion and cut with a vibratome in the coronal or parasagittal plane into 30–50-μm-thick sections that were collected serially in PBS and stored at 4 °C until processing. Every fifth section was mounted on gelatin-coated slides, air-dried and stained with Cresyl Violet. All observations were made in the first somatic sensory cortex (SI) or in the presumptive SI, that early after birth is characterized by a superficial marginal zone, a densely cellular cortical plate (CP) and a thick, less densely packed subplate region in which layers V and VI are distinguishable (Wise and Jones, 1978; Erzurumlu et al., 1990).

### Immunocytochemistry and immunoblotting

*Antibodies.* The following antisera were used: a rabbit polyclonal antibody against the N-terminus of VGAT (Chaudhry et al., 1998); a mouse monoclonal antibody against GABA (Matute and Streit, 1986); and a rabbit polyclonal antibody against the C-terminus of the rat plasma membrane GABA transporter 1, GAT-1 (Minelli et al., 1995).

*Immunocytochemistry.* For immunoperoxidase studies, free-floating sections were pretreated in 1% $H_2O_2$ (in PBS) for 30 min, rinsed in Tris 0.5 M plus 0.9% NaCl, preincubated for 1 h in 10% nonimmune newborn calf serum (NBCS) in Tris with 0.2% Triton X-100, and then incubated overnight at 4 °C in anti-VGAT primary antibody. Different working dilutions were tested (from 1:500 to 1:2000); and the best signal/noise ratio was obtained using a 1:1000 dilution. The next day, sections were incubated for 1 h in biotinylated goat anti-rabbit immunoglobulin G (IgG) (BA-1000; Vector Laboratories, Burlingame, CA, USA; 1:100 in Tris), then for 30 min in avidin–biotin–peroxidase complex (ABC Elite PK6100, Vector; 1:200 in Tris), and finally in 0.08% 3,3' diaminobenzidine tetrahydrochloride (DAB) in Tris 0.05 M with 0.02% $H_2O_2$.

For electron microscopy, sections were first rinsed in 1% sodium borohydride (in PBS) and then processed as described for light microscopy (but Triton X-100 was omitted). Sections were then osmicated in 1% osmium tetroxide and flat-embedded in Araldite. Plastic-embedded sections were studied by a correlative light and electron microscopic method described in detail elsewhere (DeFelipe and Fairén, 1993). Briefly, the sections were photographed under the light microscope and then cut again into semithin (2 μm thick) sections with a Reichert ultramicrotome. The semithin sections were examined under the light microscope and rephotographed. The selected semithin sections were resectioned at 60–70 nm and the thin sections were collected on Formvar-coated, single-slot grids, stained with uranyl acetate and lead citrate, and finally examined with a JEOL 1200 EX electron microscope (JEOL USA Inc., Peabody, MA, USA). Sections adjacent to those used for electron microscopy were Nissl-stained and used to identify the cortical layers.

For immunofluorescence, sections were rinsed in 1% sodium borohydride (in PBS), incubated in 20% NBCS in PB with 0.1% Triton X-100 for 1 h and then overnight at 4 °C in a mixture of VGAT (1:1000) and GABA (1:12000) antibodies (in 1% NBCS). The next day, sections were incubated first in 20% NBCS and then for 1 h in a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (FI-1000, Vector) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (T-2742, Molecular Probes, Eugene, OR, USA), both diluted 1:100. Double-labeled sections were examined using a BioRad Microradiance confocal laser scanning microscope (BioRad, Hemel Hempstead, UK) equipped with an argon (488 nm) and a helium/neon (543 nm) laser for excitation of FITC and TRITC, respectively. Red and green immunofluorescence were imaged sequentially and emissions were separated with 515-/30-nm band-pass (FITC) and 570-nm long-pass (TRITC) filters. Images were acquired using the 60× oil immersion lens (numerical aperture 1,4) with the confocal pinhole kept at the minimum setting (0.6–1.5 nm). To improve signal-to-noise ratio, 15 frames per image were averaged by Kalman filtering. Control sections incubated with two primary and one secondary antibody or with one primary and two secondary antisera revealed no appreciable cross-reactivity.

*Immunoblotting.* Animals were perfused with cold 4-mM Tris–HCl, pH 7.4, containing 0.32-M sucrose, 1-mM EDTA, 0.5-mM phenylmethylsulphonyl fluoride, and 0.5-mM *N*-ethylmaleimide. The cerebral cortex was excised and homogenized by glass-Teflon homogenizer in 10 vol of ice-cold homogenization buffer (0.32-M sucrose; 4-mM Tris–HCl, pH 7.4; 1-mM EDTA; and 0.25-mM dithiothreitol). Given the small size of the cerebral cortex of early postnatal animals and the consequent paucity of the protein extract we could harvest from it, for each time point studied we mixed the cortical homogenates obtained from five rats. The homogenate was centrifuged at 1000×*g* for 15 min at 4 °C. The supernatant was recentrifuged at 105,000×*g* for 1 h at 4 °C and the resulting crude membrane pellet (Thomas and McNamee, 1990) was resuspended in homogenization buffer containing protease inhibitors (leupeptine and pepstatin A, 1 μM). Protein concentrations were measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots of crude membrane fraction (containing 30 μg of protein extract) were mixed with equal volumes of 2× electrophoresis sample buffer with 4-M urea (final concentration). The samples were subjected to 7.5% sodium dodecyl sulfate-polyacrylamid gel electrophoresis with a 3% stacking gel under reducing conditions and the separated proteins were electrophoretically transferred to a 0.45-μm nitrocellulose filter (Tobwin et al., 1979). The blots were incubated with the VGAT (1:1000) or GAT-1 antibody (1:750), then with goat anti-rabbit IgG conjugated with horseradish peroxidase (PI-1000, Vector; 1:250), and finally reacted with chemoluminescence Western blotting kit (RPN 2109, Boehringer Mannheim, Mannheim, Germany). Labeled bands were visualized with BioRad Chemi-Doc; densitometric analyses were performed using the BioRad Quantity One software.

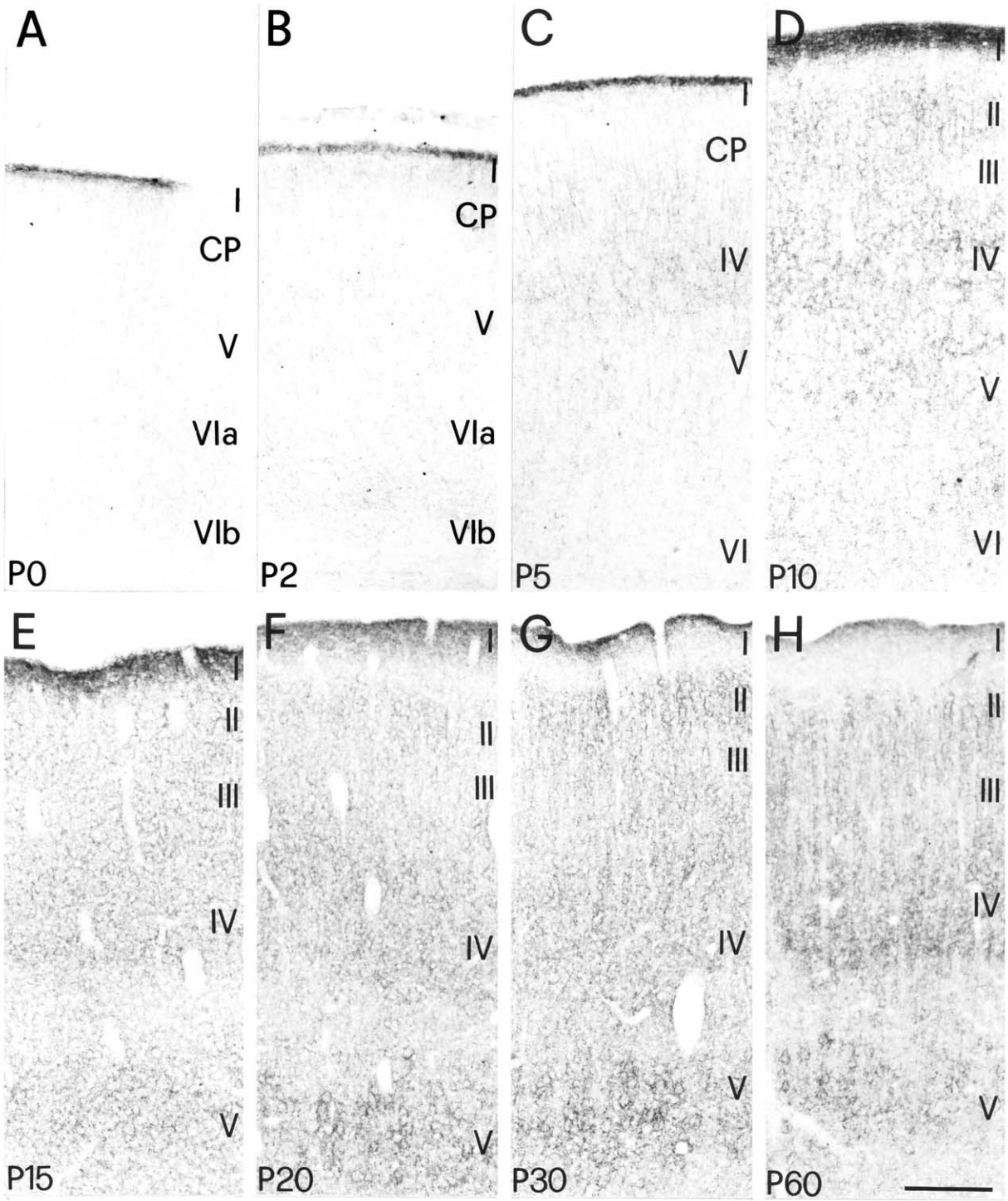


**Fig. 1.** Representative low-power photomicrographs showing the distribution of vesicular GABA transporter (VGAT) immunoreactivity (ir) in rat first somatic sensory cortex (SI) during postnatal development. VGAT ir at postnatal day (P)0 (A), P2 (B), P5 (C), P10 (D), P15 (E), P20 (F), P30 (G), and P60 (H). Roman numerals indicate cortical layers; CP, cortical plate. Bar=200 μm.

## RESULTS

### Light microscopic studies

*P0–P2.* VGAT immunoreactivity (ir) was intense in layer I, where numerous heavily stained puncta formed a continuous band (Fig. 1A, B; Fig. 2A), and weak in the rest of the cortical wall, where labeled puncta were scattered in the neuropil and slightly denser in layer VIb (Fig. 1A, B). Some fibers bearing large swellings were observed in layer V and in the CP (Fig. 2C).

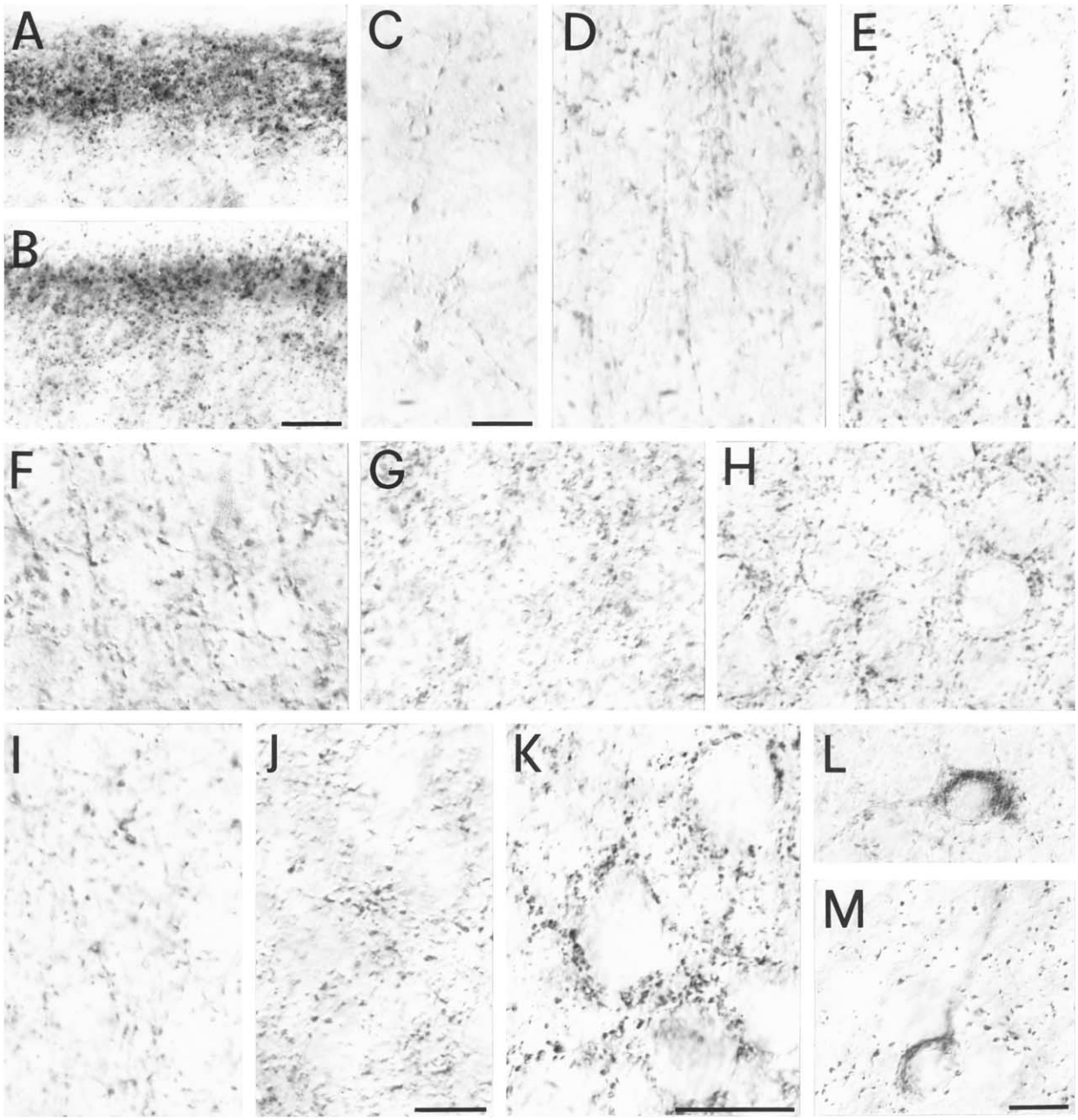


**Fig. 2.** Morphological details of maturation of vesicular GABA transporter (VGAT) immunoreactivity (ir) in the developing cerebral cortex. Layer I at postnatal day (P)0 (A) and P20 (B). Upper portion of cortical plate at P0 (C). Layers II/III at P5 (D) and P30 (E). Layer IV at P5 (F), P10 (G), and P20 (H). Layer V at P5 (I), P10 (J), and P30 (K). (L, M) Two examples of VGAT-positive cells at P10, layer V. Bars=20 μm in (B) for (A, B); 10 μm in (C); 10 μm in (J) for (D–J); 15 μm in (K); 10 μm in (M) for (L, M).

*P5.* VGAT ir was stronger than at P0–P2 (Fig. 1C). In layer I, immunoreactive puncta were tightly packed and intensely stained (Fig. 1C); in layers IV and V, numerous labeled puncta dispersed in the neuropil, rarely in apposition to unstained cell bodies (Fig. 2F, I), and fibers with intensely stained swellings were observed (Fig. 2F). In the upper CP, numerous, thin, radially oriented fibers ran toward the pia (Fig. 2D).

*P10.* Number and staining intensity of VGAT-positive puncta rose throughout the cortex: layer I displayed the highest level of VGAT ir, followed by layer IV and then by the supragranular layers and layer V (Fig. 1D). Lightly stained neurons were occasionally observed, mostly in layers V and VI: they had a fusiform or round soma and displayed one or two thick dendritic processes (Fig. 2L, M).

*P15-adult.* VGAT ir increased in layer Vb (moderately at P15 and more evidently from P20 onward) and the supragranular layers (slightly at P15–P20 and more evidently at P30), whereas it was less intense in layer I: at these stages, layers IV, Vb and upper I displayed the

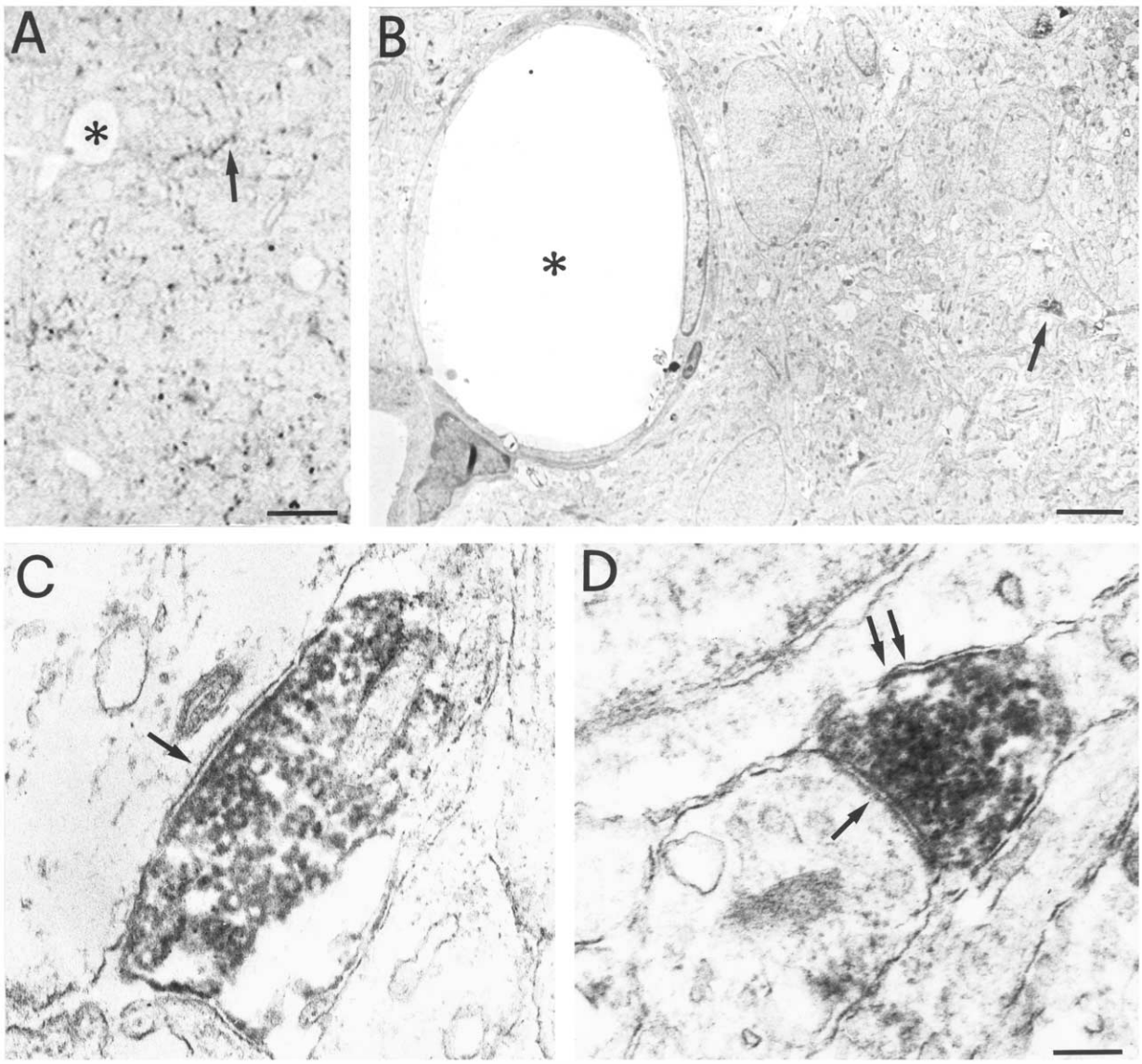


**Fig. 3.** Localization of vesicular GABA transporter (VGAT) immunoreactivity (ir) in SI at P5. (A) Semithin section from layers II/III. The arrow indicates an immunoreactive axon terminal; the asterisk a blood vessel. (B) Electron microscope image from the area illustrated in (A). Note the asterisk marking the same blood vessel. Arrow indicates a terminal of the same axon. (C) Detail of a VGAT-positive axon terminal forming a symmetric synaptic junction (arrow) with a large dendritic shaft. (D) Detail of a VGAT-positive axon terminal in close apposition to a soma, but forming a symmetric synaptic junction (single arrow) with a dendrite. Double arrow indicates a close membrane apposition superficially resembling a symmetric contact. Bar=14 μm (A); 4 μm (B); and 140 nm (C and D).

highest level of signal, followed by layers II-III (Fig. 1E–H). In all layers, but especially in Vb and IV, positive puncta were both scattered in the neuropil and around unlabeled somata and their proximal processes (Fig. 2H, J, K). Few VGAT-positive neurons were present (Fig. 2L, M). From P20 onward, thick, radially oriented fibers were frequently observed in layers II–III and VI (Fig. 2E). At P30, the intensity, distribution and morphology of VGAT ir were indistinguishable from those observed at P60 (Fig. 1G, H). The pattern of VGAT ir we observed at these stages is similar to that previously described in the adult neocortex (Chaudhry et al., 1998).

*Electron microscopic studies.* To investigate their nature, we examined several hundreds of VGAT-immunoreactive terminal-like puncta in the neuropil of the SI CP at P5 using correlative light and electron microscope methods (Fig. 3B). This time point was chosen as representative of VGAT ir in the first postnatal week because at this age ir was stronger.

Many VGAT-positive puncta could be identified as axon terminals filled with immunostained vesicles, whereas others could not be identified. Random analysis in single sections of VGAT-positive puncta identified as axon terminals revealed that most of them appeared not to have

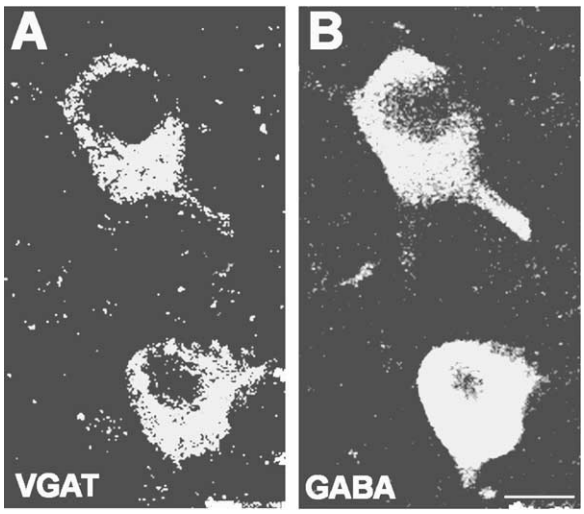


**Fig. 4.** Vesicular GABA transporter (VGAT)-positive cells contain GABA. Single-plane confocal images showing two cortical neurons in layer V (postnatal day [P]10) expressing both VGAT (A) and GABA (B) immunoreactivity. Bar=5 μm.

formed synapses, whereas the remaining formed clearly identifiable synaptic junctions.

In all VGAT-positive puncta that formed clearly identified synapses, the postsynaptic density was very thin (Fig. 3C, D), which is typical of GABAergic symmetric synaptic junctions (Peters et al., 1991; also see Micheva and Beaulieu, 1996; and DeFelipe et al., 1997). Postsynaptic dendritic profiles included large dendritic shafts (probably apical dendrites), small dendritic shafts and dendritic spines. Axon terminals forming synapses with the somata of pyramidal cells were also seen, but they were relatively few at this age.

*Colocalization studies.* Whereas at P0–P5 VGAT ir was localized only to puncta and fibers, from P10 onward it was consistently observed also in a few neuronal cell bodies, whose size and morphology were reminescent of GABAergic nonpyramidal neurons. To assess whether VGAT-positive cells contained GABA, we performed double-labeling studies at P10, P15, and P20 using GABA antibody in combination with VGAT antiserum.

In double-labeled sections, VGAT ir was as in DAB-stained material and allowed the identification of few positive fusiform or round neurons; GABA-positive neurons were uniformly distributed throughout the cortex and morphologically heterogeneous (Del Rio et al., 1992; Micheva and Beaulieu, 1995; De Felipe et al., 1997). All VGAT-positive neurons (164 from SI of two rats at each time point studied) were also GABA immunoreactive (Fig. 4), indicating that VGAT-positive neurons are GABAergic.

*Immunoblotting studies.* To obtain semiquantitative data on the variations of VGAT expression in the developing neocortex, we performed Western blottings at the same time points studied with immunocytochemistry.

At all ages, we detected a band of the apparent molecular weight of 55–60 kDa, consistent with that reported in adult whole brain extracts (Chaudhry et al., 1998; Dumoulin et al., 1999). The intensity of this band was weak at P0, it increased progressively until P15 and remained relatively stable at later ages (Fig. 5A). The relative intensity of the 55-kDa band (expressed as percentage of the adult levels) was 17% at P0, 35% at P2, 44% at P5, 68% at P10, 97% at P15, 96.5% at P20, and 101.5% at P30 (Fig. 5A).

In addition, we detected a band of approximately 220 kDa, which displayed the highest intensity at P0–P5, decreased with maturation and was barely detectable after P20 (Fig. 5A). The possibility that this band might reflect a different protein cross-reacting with VGAT antibody seems unlikely, since: (1) the morphology and distribution of VGAT+ elements show a high degree of correspondence with other GABAergic presynaptic markers throughout cortical maturation (see Discussion); and (2) ultrastructural studies at P5, when immunoblots do exhibit the additional band, revealed VGAT ir exclusively in axon terminals that never formed asymmetric contacts. Alternatively, this heavy band may correspond to a tetramerized form of a VGAT monomer or to a complex of distinct developmentally regulated proteins interacting with VGAT and regulating its trafficking (see Bedet et al., 2000). This band may thus be expression of the progressive maturation of the cortical GABAergic releasing system and reflect changes in synaptic vesicle life cycle. It is worth noting that an additional heavy band at the early and intermediate phases of brain maturation has been reported also for plasma membrane glutamate transporters (Furuta et al., 1997).

*Comparison of VGAT and GAT-1.* The distribution of VGAT ir in the developing rat neocortex closely matches that of the plasma membrane transporter GAT-1 (Yan et al., 1997; Minelli and Conti, 1999), suggesting that the two GABA transporters share similar developmental profiles. To gain further information, we used Western blotting to evaluate in the same material the relative amount of GAT-1 (expressed as percentage of adult levels) at the same time points at which we studied VGAT expression. At all postnatal ages, we detected a single band of approximately 67 kDa (Fig. 5B), in line with the estimated molecular weight of the protein (Guastella et al., 1990). The relative intensity of the band was 10.2% at birth, 24.7% at P2, 41% at P5, 84.2% at P10, 101% at P15, and 119% at P30 (Fig. 5B).

## DISCUSSION

The present immunocytochemical studies reveal that VGAT ir in the neocortex is weak at birth, it increases gradually through the first and second weeks of postnatal life and reaches the adult pattern during the third week. Immunoblotting studies of VGAT expression carried out at the same time points show a similar temporal evolution.

### During cortical development VGAT expression is coordinated with that of other GABAergic markers and slightly anticipates inhibitory synaptogenesis

The maturation of VGAT expression in the rat developing neocortex shares remarkable similarities with that of glutamic acid decarboxylase (GAD) and GAT-1. GAD activity

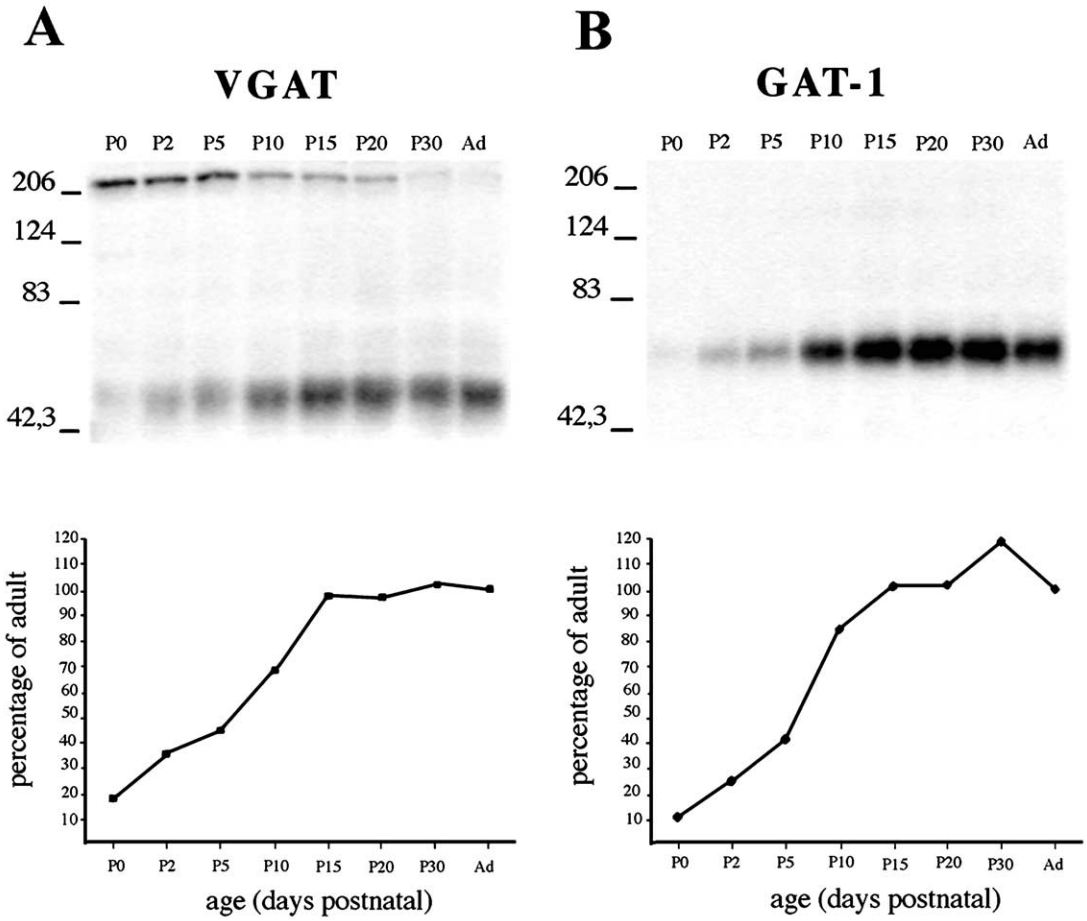


**Fig. 5.** Expression of vesicular GABA transporter (VGAT) (A) and plasma membrane GABA transporter 1 (GAT-1) (B) immunoreactivity in developing neocortex. Representative blots (top parts of A and B) showing the temporal intensity variations of immunoreactive bands migrating at approximately 55 kDa (VGAT, in A) and 67 kDa (GAT-1, in B). Note in (A) the presence of an additional VGAT-immunoreactive band of approximately 220 kDa that decreases with maturation. Reference molecular weights expressed in kilodaltons on the left of each blot. Densitometric semiquantitative analyses are reported in graphs (bottom part of A and B) showing relative immunoblot densities given as percentage of adult levels considered as 100%.

in neocortex is low at birth, it increases gradually during the first postnatal week and soars in the second and third weeks of life (McDonald et al., 1981; Wong and McGeer, 1981). The laminar distribution of both GAD- and GAT-1-positive elements at different postnatal ages (Wolff et al., 1984; Yan et al., 1997; Minelli and Conti, 1999) is similar to that of VGAT. In addition, we showed here that the developmental changes of GAT-1 and VGAT expression observed in the very same samples are quantitatively similar. Moreover, the expression of $GABA_A$ receptor $\alpha 1$ subunit, which correlates with the appearance of the physiological features of the adult type of this receptor, is low in the perinatal neocortex and increases significantly with postnatal development (Fritschy et al., 1994). Taken together, these data indicate that different proteins involved in the GABAergic synaptic function are highly coexpressed during cortical postnatal development and that their expression is regulated in a strictly coordinated manner (Eastman et al., 1999; Niehrs and Pollet, 1999).

Despite the low expression of VGAT (and the relative immaturity of inhibitory synaptogenesis; see below), GABA levels are relatively high and GABAergic neurons and receptors are present at perinatal ages (Coyle and Enna, 1976; Coyle, 1982; Lauder et al., 1986; Cobas et al., 1991; Del Rio et al., 1992; Laurie et al., 1992; Poulter et al., 1992, 1993; Fritschy et al., 1994; Vincent et al., 1995). The evident mismatch between the presence of GABAergic neurons and GABA and the lack of the molecule responsible for loading GABA into vesicles raises interesting questions on the mechanism(s) of GABA release during early postnatal life. Some evidence has been provided in favor of the hypothesis that growth cones arising from GABA-containing neurons could be the source of GABA (Taylor and Gordon-Weeks, 1991), probably by non-vesicular forms of release (e.g. by reverse transporter action; Attwell et al., 1993; but see Gao and van den Pol, 2000). In this context, it is worth noting that during the first postnatal week plasma membrane transporters GAT-1 and GAT-2 are very low or barely detectable (Yan et al., 1997; Minelli et al., 2003a; and present results), whereas the astrocytic plasma membrane GABA transporter 3 (GAT-3) (Minelli et al., 1996) is abundantly expressed in the cerebral cortex (Minelli and Conti, 1999; Minelli et al., unpublished observations). Since GAT-3 ir has been shown in GABA-con-

taining neurons (Minelli et al., 2003a) it is possible that early in development GAT-3 reverse action may mediate, at least in part, the non-vesicular release of GABA.

Inhibitory synapses are sparse at birth, when they are preferentially located in layer I and deep laminae, and their number gradually increases during the first and second postnatal weeks (Blue and Parnavelas, 1983b; Micheva and Beaulieu, 1996; DeFelipe et al., 1997). From the beginning of the third week, layers II–III, IV and V exhibit a major increase in the number of synapses and, together with layer I, they are the layers where inhibitory synapses are densest (Blue and Parnavelas, 1983b; Micheva and Beaulieu, 1996; DeFelipe et al., 1997). The present studies show that the laminar distribution of VGAT-positive puncta during cortical development follows the same spatial order as inhibitory synaptogenesis, but it is slightly anticipated. Indeed, layer IV exibits prominent VGAT immunolabeling already at P5, while layer V and supragranular layers display a substantial increase in VGAT expression between P10 and P15. In addition, immunoblot analyses reveal that VGAT expression reaches about half of the adult levels at the end of the first postnatal week, when inhibitory synapses are still few, and that the relative amount of VGAT observed at the early postnatal ages is higher than that expected on the basis of the number of inhibitory synaptic contacts reported in ultrastructural studies (Blue and Parnavelas, 1983b; DeFelipe et al., 1997). This is in line with the present observation that at P5 most VGAT-positive axon terminals did not form synaptic junctions. Taken together, these observations suggest that VGAT expression precedes the complete maturation of the ultrastructural features of inhibitory synapses.

During the period when the rate of synaptogenesis is highest, inhibitory synapses peak above adult levels (Blue and Parnavelas, 1983b; DeFelipe et al., 1997), suggesting that their pruning occurs later in cortical maturation. Other studies, however, have failed to detect a significant reduction in inhibitory synapses during late development (Micheva and Beaulieu, 1996). Whereas we observed that GAT-1 expression peaks above adult levels between P20 and P30, neither immunoblotting analyses nor immunocytochemical studies revealed obvious signs of VGAT overexpression during cortical maturation. A possible interpretation of this apparent inconsistency is that, since the number of synaptic vesicles per terminal rises progressively from birth to adulthood (Blue and Parnavelas, 1983a; DeFelipe et al., 1997), a reduction in the number of inhibitory axon terminals might be counterbalanced by an increase in the density of synaptic vesicles per terminal, resulting in a quantitatively stable expression of VGAT during late cortical development.

### VGAT may contribute to inhibitory synaptogenesis

GABA plays an important role during early development by exerting trophic influences, enhancing synaptic thickening and modulating the expression of GABA receptors (Ben-Ari et al., 1994, 2002; LoTurco et al., 1995; Belhage et al., 1998; Owens and Kriegstein, 2002). Whether these effects are mediated by paracrine or "classical" point-to-point synaptic mechanisms is still poorly understood. On the one hand, $GABA_A$-mediated currents have been recorded in proliferating/migrating cortical cells lacking anatomically defined synaptic contacts and immature cortical neurons express high-affinity, $GABA_A$ receptors that are relatively insensitive to desensitization and that increase the likelihood of activation by low levels of non-synaptically released GABA (Araki et al., 1992; Laurie et al., 1992; Ma and Barker, 1995; Poulter et al., 1993; Owens et al., 1999; Soria and Valdeolmillos, 2002). On the other, at birth many CP and subplate neurons exhibit spontaneous $GABA_A$-mediated postsynaptic currents (Owens et al., 1999; Hanganu et al., 2001) and electrical stimulation of layer I results in a $GABA_A$-mediated postsynaptic current in neonatal cortical pyramidal neurons (Dammermann et al., 2000). These data suggest that, at least in part, GABA might exert its functions by synaptic receptor activation. The presence of VGAT in fibers and axon terminals localized in the same cortical laminae where early GABAergic synaptic activity has been recorded suggests that the vesicular machinery for the synaptic GABA release is expressed at perinatal ages and may mediate the spontaneous release of GABA, which represents an essential drive for synapse formation and consolidation during early synaptogenesis.

In conclusion, the overall pattern of VGAT development in the cerebral cortex is compatible with the notion that VGAT represents a good indicator of the functional maturation of GABAergic synapses. However, the observation that VGAT maturation preceeds inhibitory synaptogenesis suggests that this transporter may contribute to the transition from a functional state dominated by the trophic role of paracrine GABA signaling, which is typical of early postnatal development, to one characterized by classical point-to-point synaptic communication, which is typical of the more mature (including adult) cortex and in which GABA becomes the main inhibitory neurotransmitter.

*Acknowledgements—This work was supported by a grant from MIUR (COFIN01) to F.C. We are grateful to Carlos Matute for the GABA antibody and Enrico Cherubini for critical comments on an earlier version of this paper.*

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*(Accepted 24 October 2002)*