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Research report

Postnatal expression pattern of calcium-binding proteins in organotypic thalamic cultures and in the dorsal thalamus in vivo

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Abstract

The present study describes the postnatal expression of calbindin, calretinin and parvalbumin and glutamic acid decarboxylase (GAD) and microtubule-associated protein 2 (MAP2) in organotypic monocultures of rat dorsal thalamus compared to the thalamus in vivo. Cultures were maintained for up to 7 weeks. Cortex-conditioned medium improved the survival of thalamic cultures. MAP2-immunoreactive material was present in somata and dendrites of small and large-sized neurons throughout the cultures. Parvalbumin immunoreactivity was present in larger multipolar or bitufted neurons along the edge of a culture. These neurons also displayed strong parvalbumin mRNA and GAD mRNA expression, and GABA immunoreactivity. They likely corresponded to cells of the nucleus reticularis thalami. Parvalbumin mRNA, but neither parvalbumin protein nor GAD mRNA, was expressed in neurons with large somata within the explant. They likely represented relay cells. GAD mRNA, but not parvalbumin mRNA, was expressed in small neurons within the explants. Small neurons also displayed calbindin- and calretinin-immunoreactivity. The small neurons likely represented local circuit neurons. The time course of expression of the calcium-binding proteins revealed that all were present at birth with the predicted molecular weights. A low, but constant parvalbumin expression was observed in vitro without the developmental increase seen in vivo, which most likely represented parvalbumin from afferent sources. In contrast, the explantation transiently downregulated the calretinin and calbindin expression, but the neurons recovered the expression after 14 and 21 days, respectively. In conclusion, thalamic monocultures older than three weeks represent a stable neuronal network containing well differentiated neurons of the nucleus reticularis thalami, relay cells and local circuit neurons.

Keywords: Calcium-binding protein; Neuronal markers; Postnatal development; Western blot; Immunohistochemistry; In situ hybridization; Roller tube culture

1. Introduction

Organotypic cultures (OTCs) of mammalian neuronal brain areas are an established model system in neurobiology [15]. Most studies have focused on layered structures such as the cerebral cortex, hippocampus and colliculus superior. Thalamic explants, in particular, were used solely as a source of afferent input to cortical explants. Considerable efforts have been made to establish thalamocortical cocultures in order to study laminar specificity of thalamic afferents and corticofugal projections, the latter arising mainly from infragranular neurons, and cortical 'stop-cues' helping thalamic afferents to target the correct cortical layers [6,7,12,20,22,26,38,51]. Further, the role of the thalamic afferents in regulating the neurochemical architecture of the cortex has been analysed [33,34]. Although these data suggest that basic structural features must have been conserved in the thalamic explant, the differentiation processes within the thalamic cultures have not been analysed so far. The present study was undertaken to analyse the postnatal development of the rat dorsal thalamus in vitro.

Abbreviations: CaBPs, calcium-binding proteins; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-propansulfonate; DIV, days in vitro; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-Hydroxyethyl) piperazine-*N*′-2-ethane sulfonic acid; OTCs, organotypic cultures; P, postnatal day; PAGE, polyacrylamide gelelectrophoresis; PMSF, phenylmethylsulfonylfluoride; SDS, sodiumdodecylsulphate; TBS, Tris buffered saline (50 mM Tris–HCl pH 7.4, 150 mM NaCl)

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In thalamic monocultures, we investigated the developmental expression of the calcium-binding proteins (CaBPs) parvalbumin, calbindin D-28k and its nervous system specific homologue calretinin, because these proteins are well known cell type-specific anatomical markers in the rat thalamus [29]. Functional roles of CaBPs include calcium buffering [17]; contribution to repolarization of the neuronal membrane [36] and calcium-signalling to intracellular target molecules [19]. CaBPs have a complex expression pattern in the rat thalamus [42,43]. For example, the retinogeniculate system includes calretinin-positive fibers and somata in the dorsal and ventral geniculate nucleus [2]. Also parvalbumin, an abundant muscle protein [4], has a high level of expression in the thalamus, e.g., in the GABAergic neurons of the nucleus reticularis thalami [1,10,13] which project to the ventrolateral thalamus and to the dorsal geniculate nucleus [47]. Another source of parvalbumin are retinothalamic afferents and thalamic relay cell axons [28]. We further studied the expression of glutamic acid decarboxylase (GAD-67) on the mRNA level. It is the synthetic enzyme for the inhibitory transmitter GABA and thus serves as a marker for inhibitory neurons. In order to demonstrate the differentiation of the cytoskeleton during development of thalamic neurons, we evaluated the expression of the neuron-specific marker MAP2. It binds to microtubules in somata and dendrites and connects to microfilaments [40]. The developmental profile of CaBP expression was compared to in vivo using western blot analysis. An immunocytochemical characterisation in vivo was not performed with regard to the well characterized architecture of CaBP structures of the dorsal thalamus [2,3,10,13].

2. Material and methods

2.1. Preparation of animals

Pigmented Long–Evans rats at different postnatal (P) days (P2, P7, P13, P21, P30, P46 and adult) were sacrificed with an overdose of sodium pentobarbital (Nembutal®, 60 mg/kg body weight, Sanofi Ceva, Hannover, Germany) for subsequent preparation of the in vivo tissue. Newborn rats (P0) were used for preparation of OTCs, and killed by decapitation.

2.2. Preparation of OTCs

2.2.1. Preparation of OTCs from occipital cortex

The occipital cortex was dissected as described [34] according to the P0 atlas of Paxinos et al. [37]. The entorhinal and piriform cortices were cut away, and the hippocampus was removed. With a coronal cut, the frontal cortex was removed. The remaining occipital cortex was coronally sliced with a tissue chopper (McIlwain, Mickle Industries, Surrey, England) into 350 μm thick slices. They were transferred immediately into Gey's Balanced Salt Solution (GBSS; Gibco, Eggenstein, Germany) supplemented with D-glucose (Merck, Darmstadt, Germany) to a final concentration of 0.65% and allowed to recover at 7°C for 60 min. Slices with perpendicular orientation were selected under a stereo microscope and placed as monocultures on defatted, baked coverslips. The tissue was fixed on the coverslip by 10 μl of chicken plasma (Cocalico, Reamstown, PA, USA) and coagulated with 10 μl of thrombin (ICN, Meckenheim, Germany) with a final concentration of 25 U/ml. The coverslips were placed in roller tubes (Nunc, Wiesbaden, Germany), and supplied with 0.75 ml semiartificial culture medium [(2/4 Basal Medium Eagle, 1/4 Hank's Balanced Salt Solution, 1/4 inactivated horse serum, 2 mM L-glutamine (all from Gibco) and 0.65% D-glucose (Merck)]. After 2 days, 10 μl of a solution containing equal volumes of uridine, cytosine-β-D-arabino-furanoside and 5-fluorodeoxyuridine (4.4 μM final concentration; all inhibitors from Sigma, Deisenhofen, Germany) were applied for 24 h to retard glial growth. OTCs were maintained for up to 70 days in vitro (DIV). The medium was harvested every 2–3 days, and this cortex-conditioned medium was immediately diluted with normal medium (1:1) and supplemented to the thalamic monocultures.

2.2.2. Preparation of OTCs from the dorsal thalamus

The dorsal thalamus was dissected as described [33] according to Paxinos [[34]: figures 98–106] into 350 μm thick frontal slices, and immediately transferred to a culture dish containing GBSS supplemented with antagonists of excitatory amino acid receptors (50 μM 2-amino-5-phosphonovaleric acid and 3 mM kynurenic acid; both from Sigma; 0.65% glucose; 2.5 mM Na_2HPO_4 pH 7.5). The subsequent procedure was as described above. The cortex-conditioned medium was fed to thalamic OTCs for the entire cultivation period for up to 7 weeks.

2.3. Immunohistochemistry

For immunohistochemistry [34], OTCs were twice rinsed in 0.1 M sodium phosphate buffer pH 7.4 and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 3 h. OTCs were then incubated for 5 min in 1% H_2O_2, followed by a treatment of 0.3% Tween 20 and 5% normal goat serum (blocking solution) for 3 h (chemicals from Sigma). Primary antibodies were incubated in blocking solution overnight at 4°C. The following primary antibodies were used: mouse-anti-calbindin (1/1000 for histochemistry; 1/2000 for protein blots; Sigma); rabbit-anti-calretinin (1/3000 for histochemistry; 1/4000 for protein blots; Swant, Bellinzona, Switzerland); rabbit-anti-

parvalbumin (1/1000, Swant); mouse-anti-MAP 2a/b (hybridoma supernatant- dilution: 1/10; Riederer and Matus [40]) and rabbit-anti-GABA (1/1000; Sigma). Biotinylated secondary antibody diluted in blocking solution (1/200) was incubated for 2 h, followed by avidin–biotin–horseradish peroxidase complex (Dakopatts, Hamburg, Germany). OTCs were rinsed for 3×15 min between incubation steps. Peroxidase reactivity was developed with 0.02% diaminobenzidine and 0.002% H_2O_2 in 50 mM Tris buffer (pH 7.4) for 10 min. OTCs were dehydrated, cleared, and coverslipped with DePeX® (Serva, Heidelberg, Germany). All antibodies yielded the widely reported staining patterns, e.g., in sections of rat cortex. Controls: Incubations without the primary antibodies remained negative.

2.4. Western blotting procedures

Thalamic tissue extracted from rats at postnatal days P0–P46 (in vivo) and from thalamic OTCs (8–46 DIV; per stage we pooled 3–5 OTCs) was homogenized in a buffer containing protease inhibitor (1% 3-[(3-cholamidopropyl)-dimethylammonio]-propansulfonate (CHAPS) (Merck); 75 mM KCl, 10 mM HEPES (*N*-(2-Hydroxyethyl) piperazine-*N*′-2-ethane sulfonic acid) pH 7.8; 0.2 mg/ml aprotinin; 1 mM PMSF (phenylmethylsulfonylfluoride); 1 mM EDTA (ethylenediaminetetraacetic acid) (all from Sigma)). The homogenate was centrifuged for 10 min at $3000 \times g$, and the pellet was discarded. The protein concentration of the supernatants was determined according to Bradford [8]. Per lane, between 50–150 μg protein were lyophilised and dissolved in SDS-sample buffer [according to Ref. [25,45], respectively] and prepared for electrophoresis. For preparation under reducing conditions, 50 mM dithiothreitol was added to the protein samples.

Electrophoresis was performed with Tris- or Tricine-containing polyacrylamide gels [25,45]. For detection of parvalbumin a 7.5–20% gradient gel was used, and linear 16.5% gels were used for calretinin and calbindin. After the protein transfer [48] for 20 h at 250 mA const. (Multiphor chamber, Pharmacia, Freiburg, Germany), the nitrocellulose (0.45 μm from Schleicher and Schuell, Germany) was blocked with 0.2% Tween 20 and 1% bovine serum albumin (BSA, Sigma) in Tris buffered saline (TBS) for 2 h. The primary antibodies for parvalbumin, calbindin and calretinin (dilutions given above) were incubated in 0.2% Tween 20; 0.7% BSA; 0.7 M D-(+)-glucose, 7% glycerol in TBS [5] overnight at 4°C. After washes, alkaline phosphatase-conjugated secondaries (goat anti-rabbit antibody; rabbit anti-mouse antibody; dilution: 1/4000; antibodies from Dakopatts) were used, and phosphatase activity was detected with 0.45% nitroblue tetrazolium and 0.35% 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roth, Karlsruhe, Germany) in reaction buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris–HCl pH 9.5).

For comparative analyses of protein amounts on western blots different samples were tested on the same blot. All investigated markers were run at least three times on sodiumdodecylsulphate-polyacrylamide gelelectrophoresis (SDS-PAGE). Controls: In order to exclude antibody crossreactivity against components of cultivated plasma clots, coagulated chicken plasma was cultivated without tissue for several days. After harvesting, plasma clots were prepared for western blotting and served as negative controls (lanes labeled 'clot' in Figs. 2–4).

2.5. Non-radioactive in situ hybridization

OTCs aged 20–25 DIV were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 2–3 h, were then rinsed in sterile phosphate buffer and processed for in situ hybridization according to Obst and Wahle [34]. The GAD-67 cDNA of 2.3 kb length (gift of Dr. Kaufman; see Ref. [33]) was cloned into pBS, and linearized with *Bam*H1 for antisense transcription starting with the T7 promoter. The parvalbumin cDNA of 0.24 kb length (gift of Dr. Berchthold; see Ref. [46]) was cloned in pGEM3, and linearized with *Hin*dIII for antisense transcription from the T7 promoter. The cRNAs [4] were transcribed in the presence of biotin-UTP (GAD cRNA) and digoxygenin-UTP (PARV cRNA) (DIG system, Boehringer, Mannheim, Germany). For double-labelling, probes were mixed at empirically determined optimal dilutions. After prehybridizing the OTCs, probes were applied for 16 h at 55°C. After stringent washes [[34]; $2 \times$ SSC, $2 \times$ SSC/50% formamide, $0.1 \times$ SSC/50% formamide, $0.1 \times$ SSC; all at 55°C for 20 min each wash], unspecific antibody binding was prevented by DIG blocking solution (Boehringer) including 2% normal goat serum (Dakopatts), followed by a mixture of alkaline phosphatase-conjugated sheep-anti-DIG F(ab)2 fragments (1/2000; Boehringer) and mouse-anti-biotin antibody (1/300; Sigma) in TBS with 0.05% Triton X-100 for 12 h at 8°C. After washes in TBS, OTCs were incubated in a biotinylated goat-anti-mouse antibody (1/300 in TBS with 1% BSA, 2% normal goat serum, 0.01% Triton X-100, Dakopatts) for 5 h at room temperature. Thereafter, the alkaline phosphatase reaction product was developed with BCIP/NBT as described (Obst and Wahle [34]) under microscopic control. Then, OTCs were rinsed in TBS and incubated in Texas Red-conjugated avidin (1/100 in TBS with 1% BSA, Serva) for 1 h at room temperature. After thorough rinses in TBS, cultures were mounted, coverslipped with immunofluore (ICN), and analyzed with standard rhodamine filters and brightfield on a Zeiss Axiophot microscope. Photomicrographs were taken with AGFA-Pan APX 100 film, and 400 ASA films for the fluorescence.

Controls: Both probes labeled distinct sets of neurons when analysed separately (on OTCs and on sections of adult rat thalamus; not shown). Hybridizations with the sense cRNAs remained negative. Omitting the labeled cRNA probes from the reaction yielded negative results. The secondaries alone gave no detectable signals.

3. Results

3.1. Histological features of thalamic OTCs

During the first 10 DIV, thalamic OTCs flattened comparatively more than OTCs of cortex. From the third week onwards, fields of differentiated neuronal somata were present, and in many cases the cell-dense medial habenular nucleus stood out on the former dorsomedial corner of the explant with visual thalamus in the opposite corner (Fig. 1A). At that stage, the cells had settled into a fairly homogenous layer, which was generally thinner than cortical cultures of the same age. Usually we observed a thickness of two-three cell body layers which however may vary within a culture. Thionin-stained cultures displayed neurons of differentiated appearance and a variety of shapes. Due to expected dissolution of axon fascicles of intrathalamic fiber tracts during the first two weeks in vitro, fields of minor cell density occurred which were invaded by neurons or glia cells from more intact parts

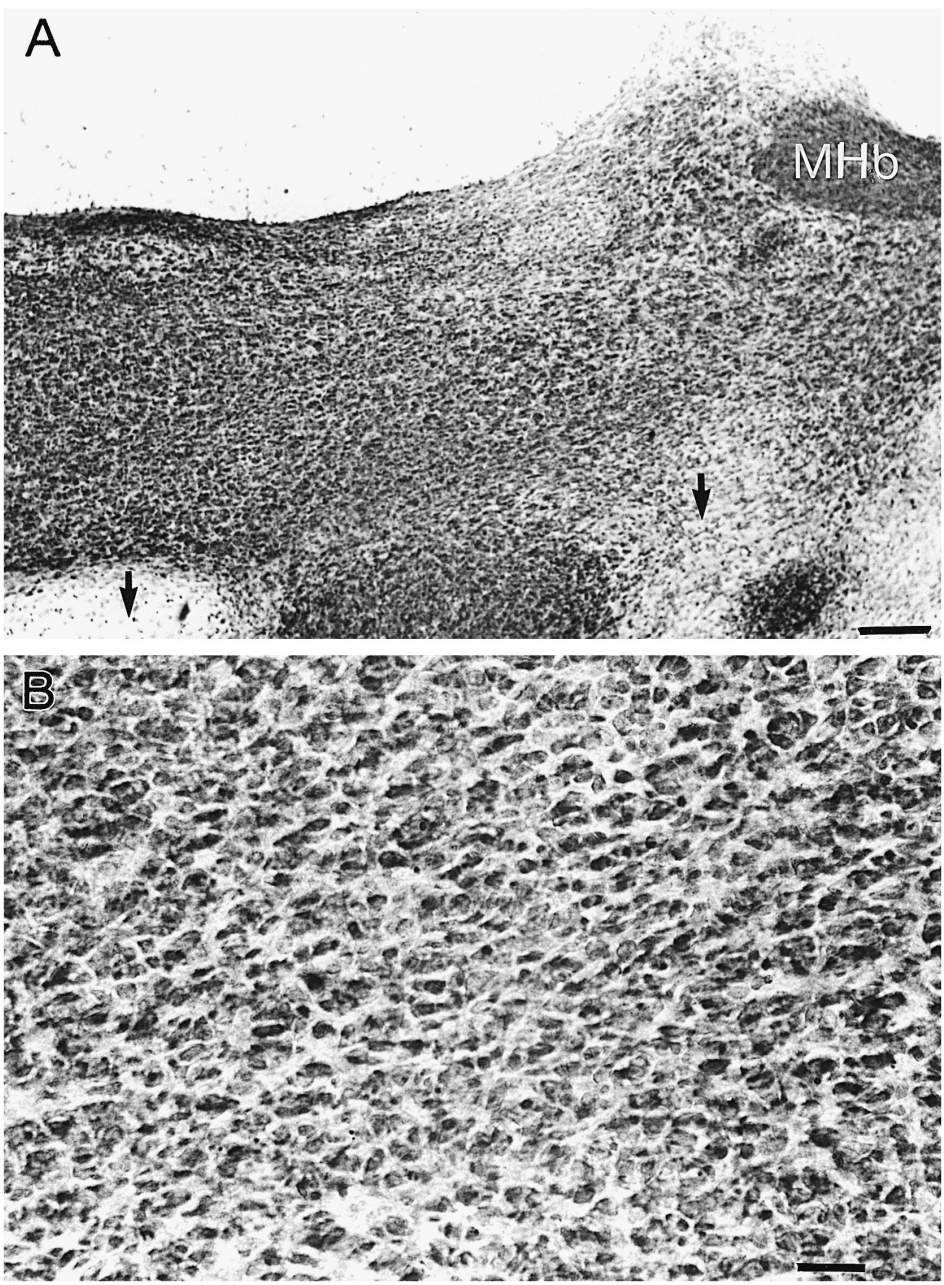

Fig. 1. Thionin-staining of a dorsal thalamus OTC after 14 DIV. Note the compact medial habenular nucleus (MHb) which was used as a landmark for orientation of the thalamic slice during preparation. The arrows in (A) point to regions of dissociated appearance with few cell bodies presumed to represent regions where axon fascicles had degenerated. (B) At higher magnification an ordered cytoarchitecture is displayed by more densely packed zones, suggestive of remnants of thalamic nuclei. Note the absence of pycnotic nuclei. Scale bar: 250 μm in A; 100 μm in B.

thus giving the impression of a locally dissociated culture. The fibers of the capsula interna/optic tract also disappeared, and the large neurons from the thalamic reticular nucleus partly embedded within the fiber tracts either became compressed into cell strands or islands along the former lateral edge of the explants or intermingled with neurons of lateral thalamic nuclei in the process of tissue flattening. Pycnotic nuclei were frequent during the first two weeks, but became rare thereafter (not shown). Feeding the cortex-conditioned medium was found to improve the survival of thalamic OTCs.

3.2. Molecular differentiation of thalamic neuronal cell classes in vitro

The following sections describe the developmental expression of CaBPs on protein blots in OTCs in comparison to those in the thalamus in vivo, and the immunohistochemical staining pattern. Further, the staining patterns for GABA and the neuronal marker MAP2 is presented, and finally, data about the localization of GAD mRNA and parvalbumin mRNA. Our aim was to search for the major thalamic cell classes in OTCs.

3.2.1. Parvalbumin protein levels and morphology of parvalbumin neurons

The developmental expression of parvalbumin is shown in Fig. 2. Parvalbumin displayed the predicted molecular size of 12 kDa, verified by the binding of the polyclonal anti-parvalbumin antibody (Fig. 2A,B; a monoclonal antibody was found to be less sensitive on the blots). This was in contrast to the results of Hogan and Berman [18] for kitten visual cortex who reported two forms of parvalbumin with lower molecular weights compared to muscle parvalbumin. In the thalamus, as well as in the thalamic OTCs we only found one form of parvalbumin identical in size to muscle parvalbumin. In vivo, parvalbumin levels were low during the first two weeks, increased massively until P21 and slightly declined thereafter (Fig. 2A). Thalamic OTCs displayed a lower level of parvalbumin expression over time in vitro (Fig. 2B) which was comparable to the level observed during the second postnatal week in vivo. The increase seen at P21 and P30 in vivo was not observed at 22 and 30 DIV in vitro. Immunohistochemistry revealed the cellular correlate for parvalbumin expression. Parvalbumin-positive neurons mainly occurred along the lateral edge of the cultures and had large somata and a strong parvalbumin expression up into dendrites and axonal processes (Fig. 2C). The dendritic organization was multipolar to bitufted and resembled the recently described [35] neurons of the thalamic reticular nucleus.

3.2.2. Calbindin and calretinin expression and cellular morphology

Fig. 3 shows the calbindin expression during postnatal development. A band of about 28 kDa molecular weight was evident. In vivo, the amount of calbindin gradually increased from P0 towards a constant level at P13 (Fig. 3A). Under in vitro-conditions the calbindin expression transiently declined after explantation and reincreased until 21 DIV to reach a steady state level thereafter (Fig. 3B). The developmental upregulation was thus delayed in OTCs for about two weeks. Immunohistochemistry (Fig. 3C) revealed intensely labeled calbindin-positive neurons with small cell bodies (average diameter: 15 μm) and mostly bitufted or bipolar dendritic trees. The distal dendrites were beaded.

Calretinin was represented by a band of about 30 kDa was already present at P0 in vivo (Fig. 4A). An increase in expression was observed at P2 and P7, and levels remained constant thereafter. In vitro, the calretinin expression disappeared almost completely following explantation (Fig. 4B). The expression recovered during the second week in vitro and reached a level similar to that found in vivo. After a slight decrease at 30 DIV calretinin expression went into a steady state level. Size and shape of calretinin-positive cells (Fig. 4C) were similar to those of calbindin-positive neurons. Multipolar, bitufted and bipolar shapes were observed possessing fine dendrites of beaded appearance.

In summary, the calcium-binding proteins calbindin and calretinin had expression levels in long-term OTCs comparable to the in vivo-situation. Although the expression was transiently reduced following explantation, both markers recovered within two weeks. The expression of parvalbumin however, showed no transient reduction, but did not reach the adult levels found in vivo.

3.2.3. Expression of MAP2 and GABA

MAP2 is a neuron-specific microtubule-binding protein which occurs in somata and dendrites. Stained OTCs revealed well differentiated neurons with large somata (Fig. 5A,B, arrows) intermingled with many medium sized and small neurons. The labeled neurites formed a highly interconnected network. MAP2 is known to be present in axons during an early stage of neural differentiation. Although we cannot exclude the presence of labeled axons, we assume that a majority of neurites actually represents dendrites, mostly the fine beaded dendrites of local circuit neurons.

GABA is the major inhibitory transmitter in the nervous system. GABA-immunoreactive structures comprised heavily labeled neurons of large size and multipolar to bitufted dendritic trees (Fig. 5C) along the edges of the culture, and less intensely labeled, smaller neurons within the explants. A dense network of labeled processes was present throughout the culture. The processes give rise to perisomatically arranged boutons which appear to outline neighboring non-GABAergic neurons (Fig. 5C).

3.2.4. Identification of interneurons and projection neurons

Having established the general histology and expression profiles of neuronal markers, OTCs were probed for the

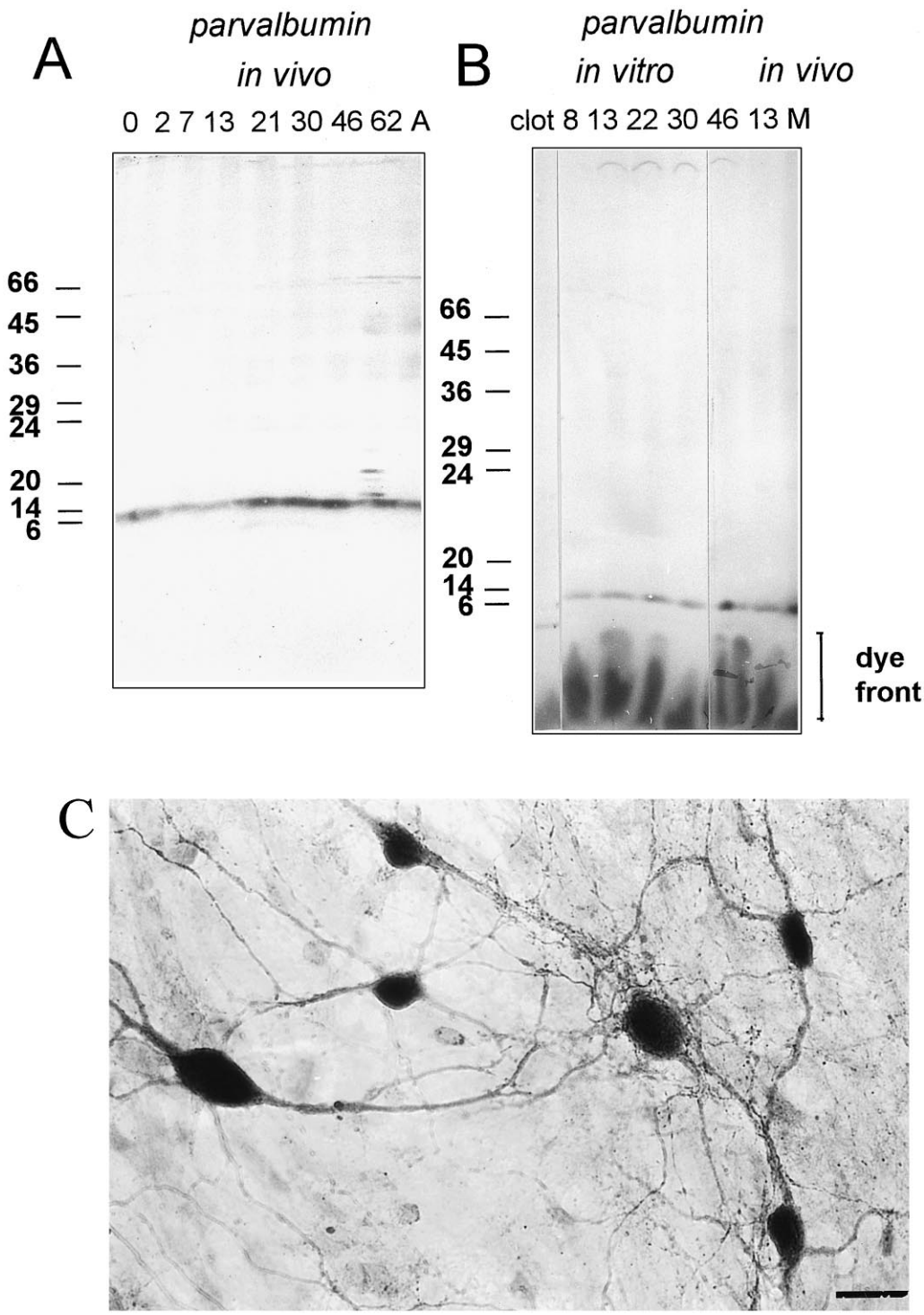

Fig. 2. Expression pattern of parvalbumin over age in vivo (A), in vitro (B), and morphology of parvalbumin-immunoreactive cells in OTCs (C). Numbers above the gel lanes indicate the postnatal age in days (A = adult). The lane labeled 'M' displayed parvalbumin reactivity in a protein fraction from rat skeletal muscle (17 μg protein loaded). The protein samples in (B) contained the tracking dye Coomassie-G which irreversibly binds to the nitrocellulose (indicated by 'dye front'). On the left side of the figures are the molecular weights (in kDa) of the marker proteins. Proteins were separated under non-reducing conditions because reducing conditions gave no immunological signal. A total of 150 μg total protein was loaded per lane. (C) Parvalbumin-immunoreactive neurons had large cell bodies of multipolar or bitufted form (8 DIV OTC). Scale bar: 30 μm.

presence of major cell classes using in situ hybridization for GAD and for parvalbumin mRNA. GAD is known to be expressed in small interneurons in some thalamic nuclei and in neurons of the perigeniculate and thalamic reticular nucleus [3,28]. Parvalbumin is present in neurons of the thalamic reticular nucleus and in thalamocortical projec-

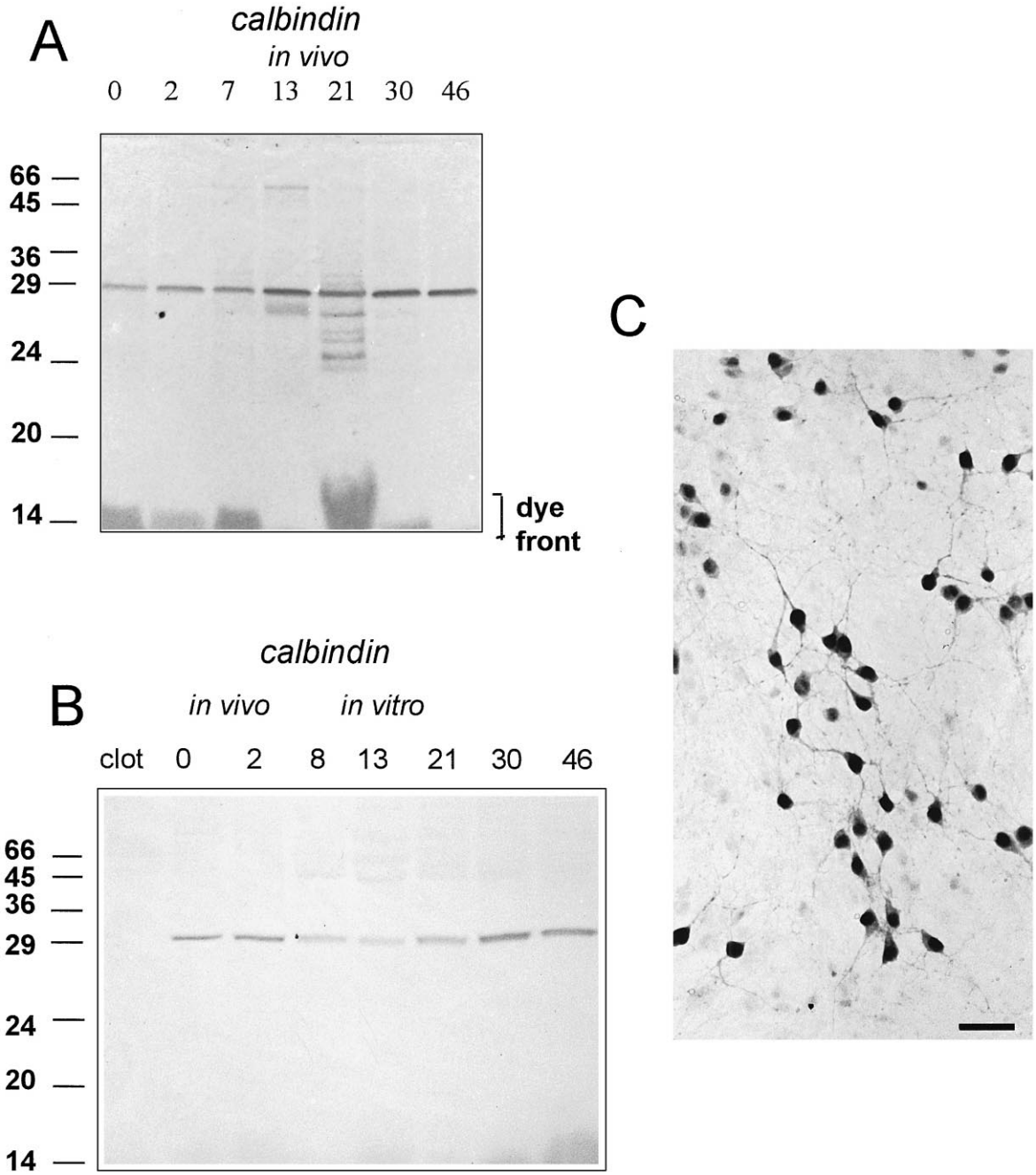

Fig. 3. Expression pattern of calbindin over age in vivo (A), in vitro (B), and morphology of calbindin-immunoreactive cells in OTCs (C) Note in (B) the reduced calbindin expression at 8 and 13 DIV. Molecular weights of the marker proteins in kDa are indicated, and the dye-front is visible in (A). Per lane 75 μg total protein was loaded. (C) Intensely stained calbindin-immunoreactive bipolar to multipolar cells (25 DIV) gave rise to a dense network of neurites. Scale bar: 50 μm.

tion neurons [10,29]. In the centre of the OTCs large parvalbumin mRNA expressing neurons were observed either as small groups or dense accumulations of cells (Fig. 6A). Smaller GAD mRNA expressing cells (Fig. 6B,C), which were negative for parvalbumin were often intermingled. Only occasionally did a large parvalbumin expressing cell display GAD mRNA (Fig. 6C,D). In contrast, along the lateral edge of the cultures, we observed zones containing numerous large, round to fusiform double-labeled cells expressing both, parvalbumin mRNA and GAD mRNA. Especially GAD mRNA was heavily expressed, and in situ reaction product could sometimes be followed into neurites extending from the labeled somata (Fig. 6D,E). In the border zones, only a few smaller-sized round GAD mRNA expressing neurons were found which were always negative for parvalbumin (Fig. 6E). The GAD mRNA expres-

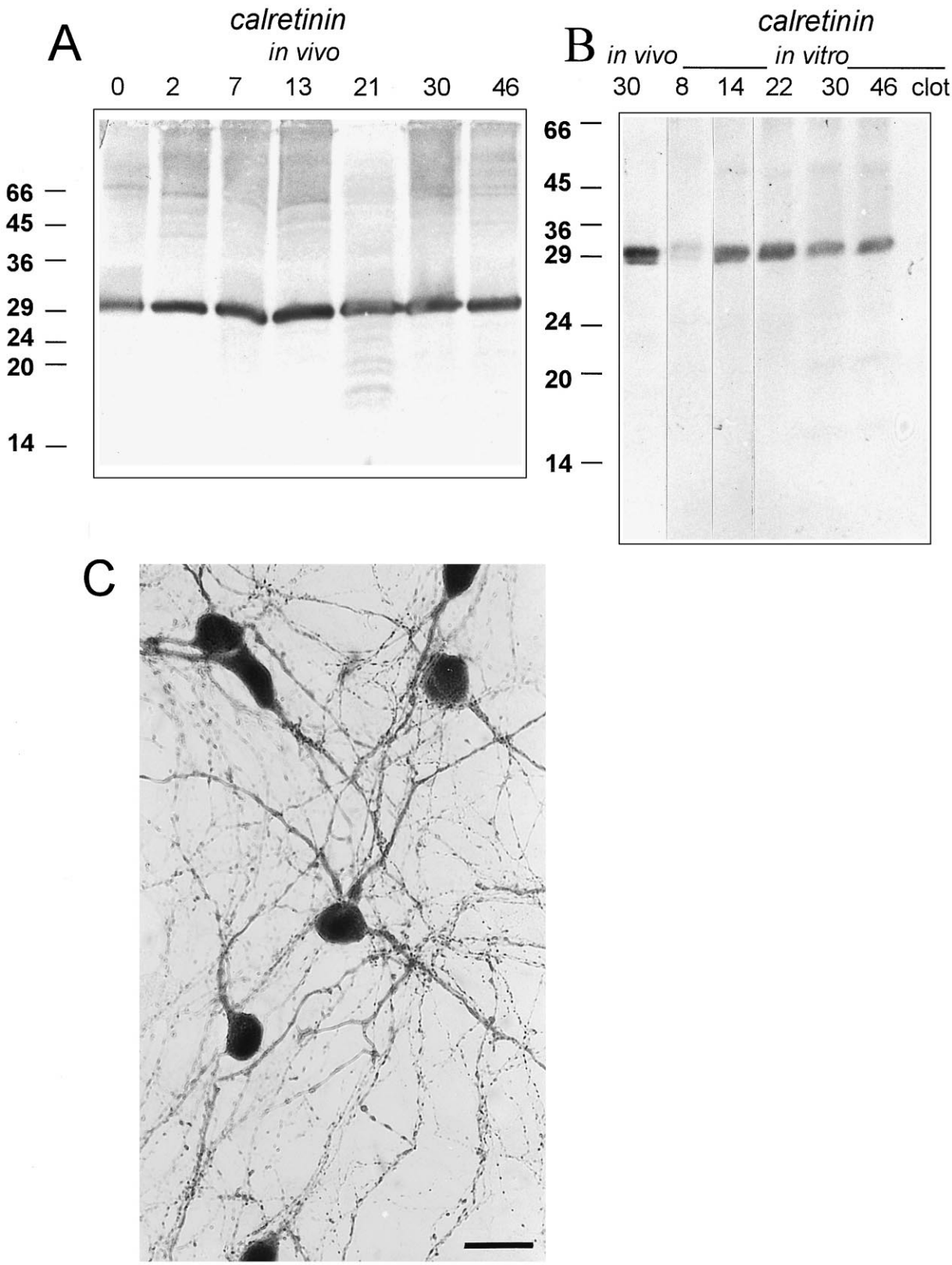

Fig. 4. Expression pattern of calretinin over age in vivo (A), in vitro (B), and morphology of calretinin-immunoreactive cells in OTCs (C). Note in (B) that calretinin was absent at 8 DIV, but has fully recovered at 14 DIV. Proteins (75 μg loaded per lane) were separated under non-reducing conditions because reducing conditions gave no signal. (C) Intensely labeled calretinin-positive cells and beaded neurites at 30 DIV. Scale bar: 30 μm.

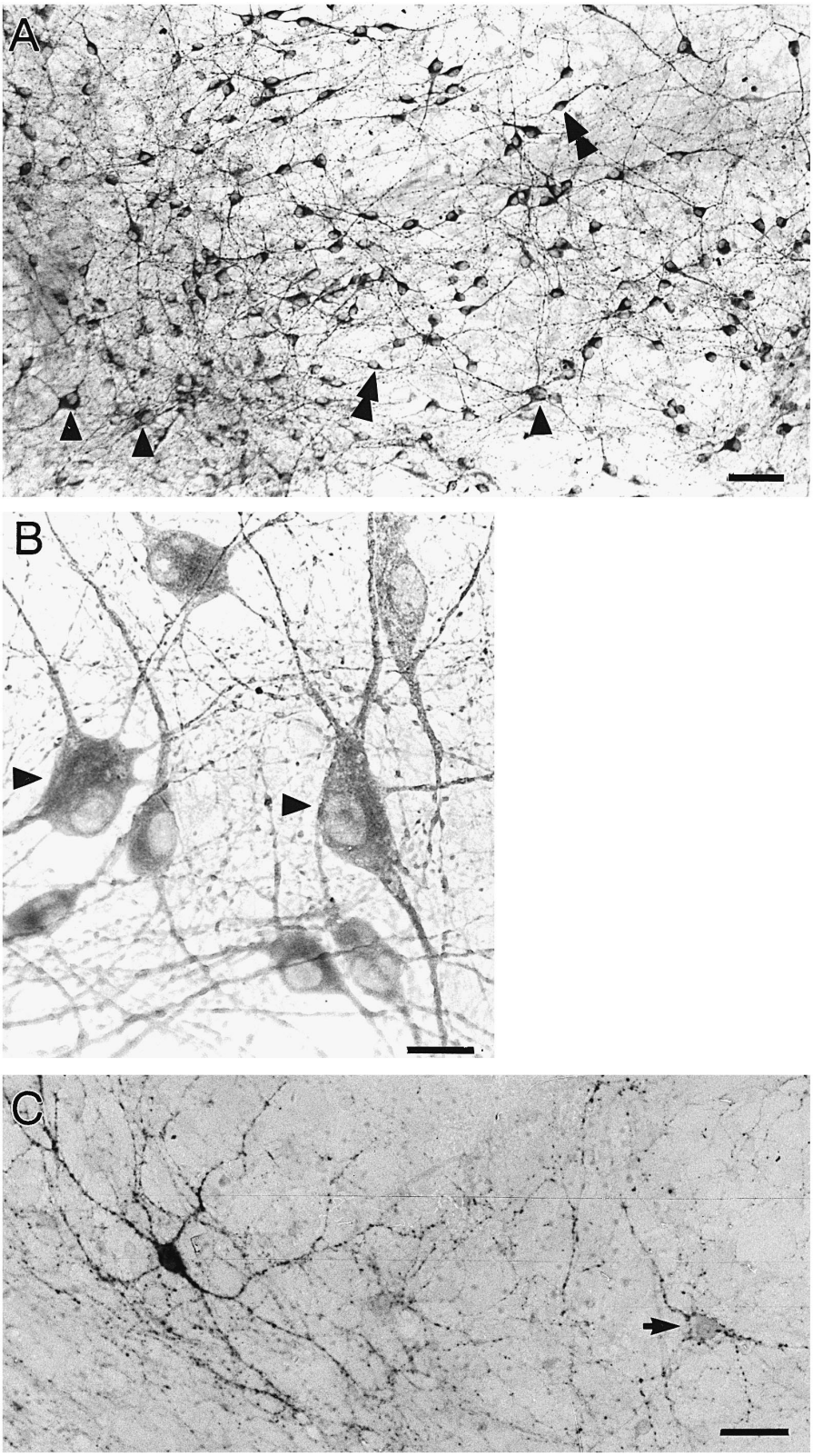

Fig. 5. MAP2-immunoreactive neurons (20 DIV) with long processes were distributed throughout the OTC (A,B). In A and B, single arrowheads indicate large multipolar cells. Double arrowheads in (A) indicate smaller bipolar cells. (B) Note the well structured appearance of neurons. (C) A heavily labeled GABA-positive neuron, and another neuron densely covered with GABAergic boutons (arrow). Numerous GABAergic puncta cover the OTC. Scale bars: 100 μm in A; 30 μm in B; 40 μm in C.

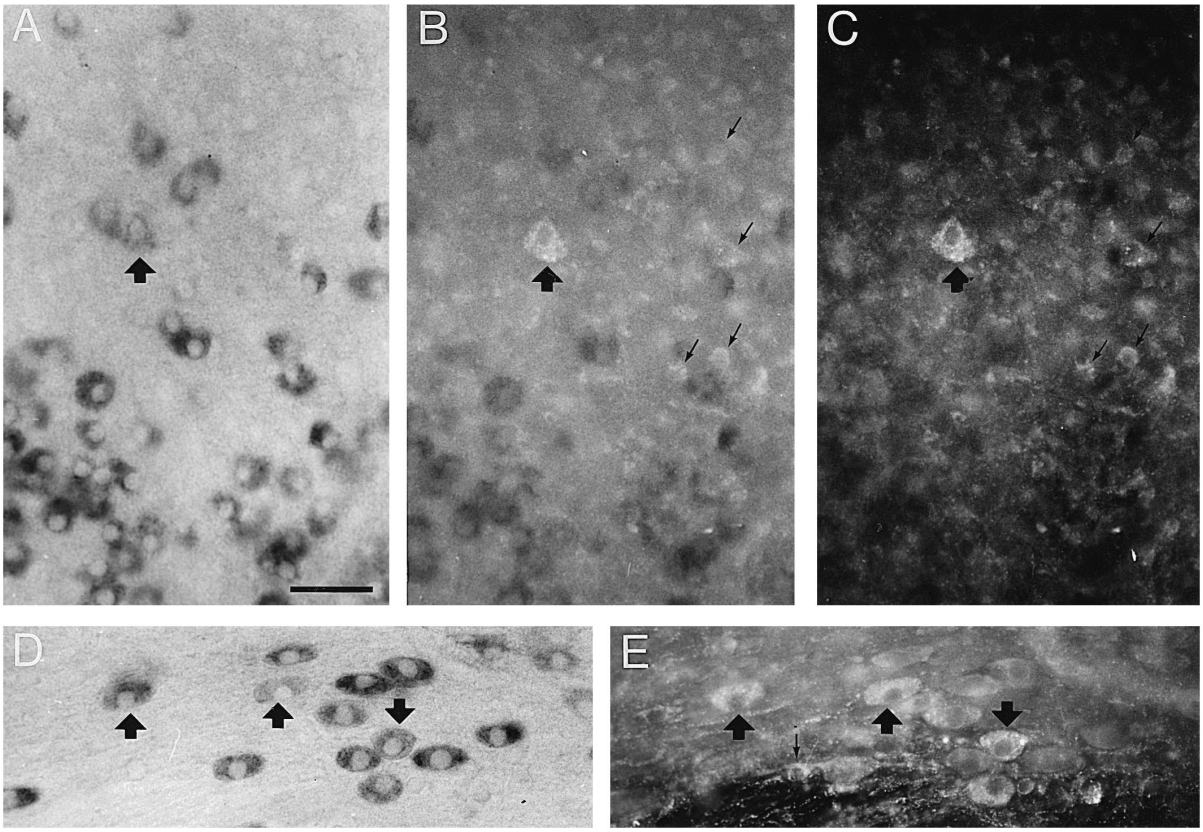

Fig. 6. Double-labelling of parvalbumin mRNA and GAD mRNA expression in OTCs. (A) Brightfield exposure shows numerous large parvalbumin expressing neurons in the centre of the culture. (B) Mixed exposure reveals one double-labeled neuron (large arrow in A–C), most likely a displaced neuron of the reticular formation. (C) Fluorescence exposure reveals smaller GAD mRNA expressing neurons (smaller arrows) intermingled with the large parvalbumin neurons, but negative for parvalbumin mRNA. (D) Along the margins of a culture a field of large fusiform parvalbuminergic neurons is present, and (E), many cells coexpressed GAD mRNA (large arrows in D,E). A few small GAD mRNA expressing neurons were negative (small arrow) for parvalbumin (E). Scale bar: 50 μm for A–E.

sion correlated with the GABA immunoreactivity which also revealed heavily labeled cells along the edges of a culture.

4. Discussion

The present study provides evidence that the mammalian thalamus can be maintained in organotypic monoculture for several weeks. So far, the isolated thalamus was cultured as an explant only for about 1 week [27,30] a period of time that covers basically the initial processes of recovery and reorganisation following explantation. Generally, the thalamus was maintained as a coculture together with visual cortex either for about 2 weeks [6,32,39,50], or as long-term coculture for up to 3 months [33]. We have now shown the ability of dorsal thalamic neurons to survive and differentiate in long-term organotypic monocultures.

Due to the 3D-organization into distinct nuclei that are separated by afferent and efferent fiber tracts, many neurons and possibly myelinating glia cells in an early stage of differentiation are traumatised during explantation and undergo cell death as is indicated by many pyknotic nuclei during the first two weeks of cultivation. After that period, the cultures usually acquires a stable state (Fig. 1A,B) for up to 7 weeks in the present study and as thalamocortical cocultures for up to 3 months in a previous study [33]. The most likely component improving thalamic cell survival is the cortex-conditioned medium. It improves the histological appearance suggesting a positive effect for neuronal survival under deafferented and deefferented conditions. The presence of such factors is most likely responsible for the long-term survival in the cocultures. Several authors have postulated that diffusible cortex-derived factors support neurite outgrowth and prevent cell death [11,26,27] although the factors have not yet been identified. Since an uptake and transport of survival-promoting agents via thalamocortical axons is not possible in monocultures, the receptors for these factors must also exist on the neuronal somata.

The explantation thus influences the thalamus in a more dramatic way than a layered cortical structure which at birth is largely perpendicularly organised with regard to

apical dendrites of principal cells and the immaturity of inhibitory cells. In cortex explants, neurons situated in the damaged surface of the slices die during the first week in vitro but the remaining cells regenerate, grow dendrites and survive due to their rich intrinsic axonal connections. In contrast, the dorsal thalamus is organized into globular nuclei of mostly projection cells which relay sensory information towards the neocortex [21]. The axons of these relay cells have already grown towards the cortical target areas before birth, and they have little connections intrinsic to the thalamus. The loss of efferent targets, and the loss of afferent innervation from sensory systems or from the neurons of the nucleus reticularis thalami which are already established at birth, represents an enormous challenge to thalamic neurons. Further, all cut fiber fascicles degenerate and likely the associated glia cells die as well. Their death may further disturb adjacent neurons fighting for survival. Therefore only a fraction of thalamic neurons survives and thalamic cultures grew much thinner than, e.g., cortex cultures. Nevertheless, many thalamic neurons of all types survive and are able to reorganize as revealed by the thionin and MAP2 staining. This is evident for the fields of rather dissociated histological appearance where neurons have settled in areas formerly occupied by fiber fascicles. The neurons have sprouted a rich network of dendrites and axons. GABAergic axons and boutons have grown over the entire culture. In rodents, most of the inhibition in thalamic nuclei is derived from inhibitory reticular and perireticular neurons [24,44] which in part start to mature prenatally [31]. Besides, the geniculate nuclei possess intrinsic GABAergic cells [14] which are still immature at birth. The presence of inhibitory neurons within the explants suggests that the thalamic interneurons continue to differentiate in vitro, and they innervate thalamic neurons with boutons highly suggestive of synaptic contacts. These inhibitory connections are assumed to be organotypic. However, due to a lack of topographical cues the reticular projection in vitro remains most likely as diffuse as in early developmental stages in vivo [31]. Further, we assume that all thalamic neurons in monocultures continue to develop synapses, because synaptogenesis in the thalamus is largely a postnatal process. Newly forming reorganized synaptic circuits in long-term monocultures likely include aberrant components, which may compensate for the loss of afferent synapses and efferent connectivity.

Thalamic neurons in vitro acquire or continue to express a set of functional markers, especially CaBPs, in a cell type-specific manner. The calbindin and calretinin-positive neurons in OTCs resemble multipolar to bipolar local circuit neurons with small somata described in thalamic nuclei and the thalamic reticular nucleus in vivo [3,10,13,43]. Calbindin and calretinin are already expressed at birth, and increase continuously during development in vivo. Surprisingly, they decline dramatically in the explants. This could suggest that the calbindin and calretinin expression in thalamic neurons is controlled by afferents. Expression declines upon deafferentation, but lateron recovers either by cell-autonomous mechanisms or by developing intrinsic connections which compensate for the loss of afferents and reactivate calbindin and calretinin synthesis. Alternatively, calbindin and calretinin expression depend on activity, and activity most likely declines upon explantation, but recovers during subsequent reorganisation and synaptogenesis in vitro.

The onset of parvalbumin expression in the dorsal thalamus is also most likely prenatal, because it is evident by embryonic day 18 in the thalamic reticular nucleus [47]. On protein blots, parvalbumin was present from the earliest stage analysed onwards. Frassoni et al. [13] described an increase of parvalbumin expression between P0 and P21. Our study now shows that this increase occurs during the third postnatal week in vivo. It could reflect the maturation of afferent fiber systems. For instance, retinal ganglion cells express parvalbumin mRNA and the protein is mostly exported into the retinothalamic and retinotectal axons [49]. However, this projection contains parvalbumin already in the new-born. Other thalamic afferents in vivo may acquire or increase parvalbumin expression, and since those are lacking in OTCs, the cultures do not display the parvalbumin increase. Another possible explanation for the reduced parvalbumin expression in OTC is that the expression depends on activity ([9]; Wahle, unpublished observations in cortex cultures). The double-in situ hybridization has revealed many parvalbumin mRNA expressing neurons, whereas the number of parvalbumin-immunoreactive cells has always been smaller, and those present have been mostly situated along the edges of the cultures. These cells are inhibitory neurons as indicated by the coexpression of GAD mRNA. In contrast, relay cells contain no detectable parvalbumin protein in their somata, but contain parvalbumin mRNA. This could indicate that presumed relay cells transcribe parvalbumin mRNA, but in the absence of sufficient bioelectric activity or in the absence of an appropriate target region do not translate the protein. The latter possibility suggests a target influence on posttranscriptional processing, and cocultures with cortex will help to analyse this possibility. In contrast to relay cells, parvalbumin protein and its encoding mRNA is present in presumed cells of the thalamic reticular nucleus where it may be expressed in a constitutive, activity-independent way.

The findings of the double-in situ hybridization support our assumption of a survival of three major cell classes in thalamic monocultures. Firstly, the large parvalbumin mRNA and GAD mRNA expressing cells most likely correspond to neurons of the thalamic reticular nucleus which deliver inhibition to the thalamus. The parvalbumin and GABA immunostaining also identified round to fusiform large neurons along the borders of the cultures suggesting that these neurons express the parvalbumin protein and its encoding mRNA, as they do in vivo [3,10,46]. Secondly, the presence of small-sized GAD

mRNA expressing neurons suggest a survival of local interneurons known, e.g., for the LGN of the rat [14]. Thirdly, the large parvalbumin mRNA expressing cells which are often distributed in clusters suggestive of remnants of thalamic nuclei, and which were negative for GAD mRNA correspond most likely to relay (projection) neurons. The absence of GAD mRNA suggests an excitatory phenotype and rat thalamic relay cells are believed to contain glutamate [23]. Evidence that thalamocortical relay cells express parvalbumin was previously presented [16,29], and parvalbumin is present in the thalamocortical axons [1,41]. Generally, our data show that relay cells survive in monocultures. A sudden death of all relay cells upon explantation is further unlikely, because relay cells are able to regenerate the thalamocortical connection in cocultures during the first two weeks in vitro [7,33,51].

In summary, our findings indicate a surprisingly high capacity of thalamic neurons to survive and reorganize in a culture model. Due to the progredient lack of topographical organisation over age in vitro it is impossible to identify the original thalamic nuclei, except for neurons of the thalamic reticular nucleus. However, the three major thalamic cell classes could be distinguished in the OTCs, suggesting that the explantation and reorganization do not induce a loss of selected cell classes or a loss of cell type specificity in the expression of functional markers.

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