Expression of type 1 inositol 1,4,5-trisphosphate receptor during axogenesis and synaptic contact in the central and peripheral nervous system of developing rat

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SUMMARY

Release of intracellular Ca$^{2+}$ is triggered by the second messenger inositol 1,4,5-trisphosphate, which binds to the inositol 1,4,5-trisphosphate receptor and gates the opening of an intrinsic calcium channel in the endoplasmic reticulum. In order to understand the importance of this mechanism in development, we have examined the distribution of the type 1 inositol 1,4,5-trisphosphate receptor during development, in some areas of the rat brain and spinal cord and in peripheral neurons, using in situ hybridization and immunohistochemistry. In brain, we find that type 1 inositol 1,4,5-trisphosphate receptor is expressed in neurons from very early in development; low levels of expression are first detected after the neurons have migrated to their final positions, when they start to differentiate and begin axonal growth. Increasing levels of expression are observed later in development, during the time of synaptogenesis and dendritic contact. Glial cells do not express type 1 inositol 1,4,5-trisphosphate receptor, except for a transient period of expression, probably by oligodendrocytes, in developing fibre tracts during the onset of myelination. In contrast with the brain, both grey and white matter of the spinal cord express type 1 inositol 1,4,5-trisphosphate receptor throughout development, and it remains present in the adult spinal cord. We also show, for the first time, that type 1 inositol 1,4,5-trisphosphate receptor is expressed in the peripheral nervous system. Strong labelling was observed in the dorsal root ganglia and during development this expression seems to coincide with the onset of axogenesis. These results suggest that type 1 inositol 1,4,5-trisphosphate receptor may be involved in the regulatory mechanism controlling Ca$^{2+}$ levels in neurons during the periods of cell differentiation, axogenesis and synaptogenesis.

Key words: inositol trisphosphate receptor, neuronal development, intracellular calcium, rat

INTRODUCTION

Many hormones, neurotransmitters and growth factors stimulate the hydrolysis of phosphoinositol 4,5-bisphosphate, generating the two second messengers, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). The IP$_3$ binds to the IP$_3$ receptor (IP$_3$R), promoting Ca$^{2+}$ release, while DAG activates protein kinase C. The IP$_3$R is a specific intracellular calcium release channel protein, which has been identified in several cell types, such as neurons and secretory cells (Berridge, 1993). It is highly enriched in the Purkinje cells of the cerebellum (Ross et al., 1989; Mignery et al., 1989), thus facilitating its purification and cloning from mouse (Furuichi et al., 1989), rat (Mignery et al., 1990) and human tissues (Ross et al., 1991). The IP$_3$R protein is a tetrameric complex, with each subunit possessing an IP$_3$ binding site in the N-terminal domain, and a transmembrane C-terminal domain that forms the oligomeric channel (Mignery and Südhof, 1990). The primary structure of the IP$_3$R shares homology with the other intracellular Ca$^{2+}$ channel, the ryanodine receptor (Mignery et al., 1989).

A family of IP$_3$R has now been identified, generated both by differential splicing and from different genes. Currently, five distinct IP$_3$R isoforms and two splice variants of type 1 IP$_3$R (IP$_3$R1) have been described (Ross et al., 1992; Danoff et al., 1991; De Smedt et al., 1994; Südhof et al., 1991). PCR analysis suggests that the distribution of distinct isoforms and splice variants is different between tissues and that they are expressed in a developmentally regulated manner, with IP$_3$R1 being the most widely and abundantly expressed (Ross et al., 1992; Nakagawa et al., 1991). A recent study of the various types of IP$_3$R isoforms has indicated that multiple transcripts can be co-expressed in several tissues and within a single cell line (Newton et al., 1994; De Smedt et al., 1994). The heterogeneity of the IP$_3$R might explain some of the biochemical and physiological discrepancies that have been observed in different systems (Meldolesi, 1992).

To further understand the involvement of intracellular Ca$^{2+}$

in the nervous system, it is necessary to determine the localization, at the cellular and subcellular levels, of the different isoforms and splice variants of IP₃R in the brain. Early studies using autoradiography indicated the presence of IP₃R in the brain, but the limitations of the technique prevented its localization to a particular type of cell (Worley et al., 1989).

Recently, a limited analysis of the expression of IP₃R in developing mouse cerebellum and adult mouse brain (Nakanishi et al., 1991), and more detailed immunohistochemical studies in adult rat brain (Sharp et al., 1993a; Rodrigo et al., 1993), have been reported. These studies show that the IP₃R is widely distributed throughout the central nervous system in neuronal cell bodies, fibers and terminals. Expression of the IP₃R is highly enriched in Purkinje cells, and found at lower levels in the CA1 region of the hippocampus, striatum, cerebral cortex, circumventricular organs and neuroendocrine structures. However, neither immunohistochemical study used antibodies specific for a particular IP₃R isoform. Limited studies have also obtained conflicting results using specific isoform antibodies against types 1, 2 and 3 IP₃R (IP₃R1,2,3). Sharp et al. (1993b) reported IP₃R3 to be expressed in neurons and IP₃R2 in glial cells, while Yamamoto-Hino et al. (1995) reported that IP₃R3 is localized in astrocytes, ependymal and Bergmann glial cells. These discrepancies need to be resolved. In the present study we have examined the expression of IP₃R1 during development of the nervous system of the rat, using in situ hybridization and immunohistochemical analysis, and report that expression occurs during the time of axogenesis and synaptogenesis.

MATERIALS AND METHODS

Pregnancies in rats (Wistar) were dated from the appearance of a vaginal plug [embryonic day (E) 0]; rats gave birth at E21. Day of birth was taken as postnatal day (P) 0.

Immunoblot analysis

Adult rat cerebellar tissue was homogenized in 6 volumes of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and the nuclear fraction removed by a brief pulse of centrifugation (11,600 g, 5 seconds) in a microfuge. The membrane fraction was pelleted by centrifugation at 4°C (11,600 g, 30 minutes). The supernatant was stored at −70°C until it was required. Samples were denatured for 5 minutes at 95°C in non-reducing Laemmli electrophoresis sample buffer, and electrophoresed in 8% (w/v) polyacrylamide with a 3% (w/v) stacking gel (Laemmli, 1970). Proteins for immunoblot analysis were transferred (5.0 mA/cm² for 1 hour) to Immobilon P membrane (Millipore). The lane containing molecular mass markers was stained with 3% (w/v) amido black. The other part of the filter was blocked in 25% non-fat skimmed milk in 50 mM Tris-HCl, pH 7.5/150 mM NaCl (TBS) containing 0.1% NaN₃ for 1 hour, then incubated with rabbit-IP₃R1 C-terminal 19-amino-acid-peptide antisera (1:1000) (Mignery et al., 1989), in 1% non-fat skimmed milk in the same buffer at 4°C overnight. After three washes in TBS for 5 minutes, the blot was incubated with biotinylated goat anti-rabbit antibody (1:400) (Vector Laboratories) in 2.5% non-fat skimmed milk/TBS at room temperature for 1 hour and further washed in TBS. The blot was incubated with streptavidin alkaline phosphatase (1:3000) in TBS for room temperature for 1 hour (Amersham), washed three times in TBS and developed in NBT/X-phosphate (Boehringer Mannheim) in 100 mM Tris-HCl, pH 9.5/100 mM NaCl/150 mM MgCl₂ solution in a sealed bag at room temperature in the dark, overnight. The reaction was stopped by washing the blot in 10 mM EDTA, pH 8.0, several times.

In situ hybridization

IP₃R1 cDNA (nucleotides 818-1763, a gift from Dr A. Marks) cloned into Bluescript II (HindIII site) (Stratagen, La Jolla, CA, USA) was linearized with EcoRV and XhoI. Antisense and sense RNA probes were labelled with digoxigenin-UTP by transcription in vitro according to the Boehringer digoxigenin-RNA labelling kit. The basic methodology for in situ hybridization has been previously described (Lai et al., 1992). Hybridization with the riboprobe was performed at 40, 45, 50, 55 and 60°C in order to determine the highest stringency conditions that retained specific labelling of IP₃R1, and thus to eliminate the possibility of cross-hybridization with other IP₃R isoforms. All subsequent experiments were performed at the hybridization temperature of 45°C, with 55°C washes, since this was the highest temperature at which specific labelling was observed.

Immunohistochemistry

For immunohistochemistry, IP₃R1 was detected with polyclonal rabbit antibodies against the rat brain IP₃R1 C-terminal 19-amino-acid-peptide (Mignery et al., 1989) coupled to keyhole limpet hemocyanin, and affinity-purified on immobilized peptide. Animals were deeply anesthetized and perfused with 0.1 M PBS, pH 7.4. The tissues were dissected out and frozen in powdered dry ice. Sagittal, horizontal and coronal frozen sections (10 mm) were mounted and dried onto gelatin-coated slides. Sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 minutes and washed in PBS. Non-specific binding sites were blocked with 10% non-fat skimmed milk powder in PBS for 3 hours prior to incubation in IP₃R1 antibody (1:1000 dilution) at 4°C overnight. After washing three times in PBS for 5 minutes, the sections were incubated with 1:200 biotinylated goat anti-rabbit antibody in PBS for 30 minutes (Vector Laboratories) and, after further washing, incubated in ABC kit (Vector Elite reagent) for 30 minutes. After additional washes in PBS, peroxidase was developed with diaminobenzidine for 3-5 minutes. The sections were counterstained with thionin, dehydrated and mounted in a dibutylphtalate/Histoclear mixture (National Diagnostics). Controls were performed by incubating the antibody with a tenfold excess of the synthetic peptide for 2 hours at room temperature, also by omitting the primary antibody or replacing with an equivalent concentration of normal rat IgG serum.

RESULTS

The rabbit antiserum against the IP₃R1-specific peptide sequence was found to recognise the appropriate single high molecular mass polypeptide of 260,000 upon western blot analysis of rat cerebellar homogenates (Fig. 1), thus confirming specificity for the rat IP₃R.

Expression of IP₃R1 in cerebellum

As previously described (Ross et al., 1989; Nakanishi et al., 1991; Sharp et al., 1993a; Rodrigo et al., 1993), IP₃R1 was expressed in Purkinje neurons most prominently compared to other areas of the adult brain, as shown by in situ hybridization (Fig. 2A,B,C) and immunohistochemistry (Fig. 2D,E). The IP₃R1 message was expressed at highest levels in the cytoplasm and dendrites (Fig. 2B), while the protein expression extended into both dendritic arborization and axons (Fig. 2D). Other calcium binding proteins have also been reported to be expressed in Purkinje cells, such as ryanodine receptors (Ellisman et al., 1990), calsequestrin (Volpe et al., 1990) and calbindin (Jande et al., 1981).

During development a group of differentiating cells expressing IP₃R1 in the dorsal region of the rostral cerebellar plate was clearly recognizable at the protein level by E14 (Fig. 3A).
eral gradient was observed in the maturation of Purkinje cells (Fig. 3F). Yet formed and their axons did not express IP3 R1 into the white matter (Fig. 3E). In contrast, in the anterior vermis. The molecular layer had increased in depth and the granular layer was apparent. The Purkinje cells were more differentiated, dispersed over the surface of the cerebellum. During the next 3 days the staining spread rostrally over the surface of the cerebellum.

In the newborn rat, the IP3 R1 was clearly expressed in all the Purkinje cells between the molecular layer and the white matter. However, the levels of expression varied regionally, depending on the plane of section. In a sagittal section, where the primary fissure was well formed, more staining was observed in the anterior vermis (Fig. 3C), but in a more lateral section the highest labelling was under the primary fissure. Anteriorly the labelling was reduced to patches, and in the hemisphere the cells had the lowest levels of IP3 R1 expression. In the posterior vermis before the secondary fissure, Purkinje cells were heavily stained. These immature Purkinje cells, which formed several (2-4) rows of cells, were not oriented and had irregular shapes. The cytoplasm of dendrites that were budding from all sides and axons, in the cells in which they could be identified, expressed IP3 R1.

An increase in the immunostaining was observed as the Purkinje cells differentiated. By P3 the Purkinje cells were all heavily labelled and the rows of cells had increased (3-5) (Fig. 3D), which presumably reflects the fact that more cells have reached the surface. Their dendrites and axons were labelled, but the axonal expression did not extend into the white matter.

The maturation of the Purkinje cells was evident by P7 and a caudal to rostral gradient was discernible. Posteriorly the Purkinje cells were more differentiated, dispersed over the cortex almost in monolayer and expressed more IP3 R1 than in the anterior vermis. The molecular layer had increased in depth and the granular layer was apparent. The Purkinje cells had primary dendrites and the IP3 R1 was present throughout axons extended into the white matter (Fig. 3E). In contrast, in the anterior vermis the Purkinje cells were just starting to differentiate and the granular layer had not yet formed. The cells were arranged in several rows; their primary dendrites were not yet formed and their axons did not express IP3 R1 into the white matter (Fig. 3F).

In addition to the caudal-rostral gradient, a ventrodorsolateral gradient was observed in the maturation of Purkinje cells by P10, with an increase in the levels of IP3 R1. The cells started to have a pear shape and the primary dendrites were enlarged and ramified. At this age all the Purkinje cells were arranged in a monolayer and all the axons projecting through the white matter into the deep cerebellar nuclei were labelled. By P14 the differentiated Purkinje cells had all reached the same levels of expression (Fig. 3G,H).

In addition to Purkinje cells, there were also a few labelled cells in the upper part of the granular cell layer by P10. Some sparsely labelled cells were observed in the granular layer using in situ hybridization (Fig. 3G). Low levels of expression was also observed at the protein level (Fig. 3H); from the position and number of cells stained they would appear to be Golgi cells.

Expression of IP3 R1 in the hippocampus

In the hippocampus we were able to detect very weak immunoreactivity of IP3 R1 as early as E20. In the newborn rat the perikarya of all the pyramidal neurons and a few dentate granule cells were labelled (Fig. 4A). As development progressed and the cells started to differentiate, higher levels of IP3 R1 were detected in all the neurons, but the levels in CA1 showed a greater increase relative to the levels in other neurons (Fig. 4B,C,D).

From P3 to P10, the outside to inside gradient of maturation that has been reported in hippocampal neurons (Zimmer, 1978) was observed in the CA1 neurons, where the more mature neurons were labelled more intensely. The expression of IP3 R1s in CA1 dendrites appeared in a rostral-caudal gradient from P7-P10, and by P14 all the dendrites from the CA1 pyramidal cells that project into the stratum radiatum and stratum lacunosum moleculare were heavily stained; as the cells differentiated the levels of IP3 R1 increased. Lower levels of expression were also observed in the dendrites of the CA2 and CA3 pyramidal cells.

In contrast to the pyramidal neurons, only a few dentate granule cells expressed low levels of IP3 R1s and this was restricted to the cytoplasm. Granular neurons are also generated in an outside to inside gradient over the period of P5-P21 and their generation continues at a low rate in adults (Altman and Bayer, 1990). IP3 R1 was expressed during PO-P7 only in the more mature cells facing the molecular layer, which was extended in a lateral to medial gradient as the dentate gyrus differentiated. By P10-P21, the granular layer was thicker and more cells were immunoreactive, although the newly generated neurons facing the hilus were not labelled (Fig. 4D, inset). This pattern of expression remained in the adult.

Expression of IP3 R1 in the neocortex

In the adult neocortex, strong expression of IP3 R1 was observed in neuronal cytoplasm and processes (Fig. 5A). The non-pyramidal and pyramidal neurons of layer II and V, respectively, were stained more heavily than the other neurons.

During development the expression of IP3 R1 was observed in the continuous line of subplate neurons, with a thin cortical plate above it also containing labelled cells by E17 (Fig. 5B). Ventricular cells express IP3 R1 mRNA from E14-E20 and in the intermediate zone very few cells, whose morphology and position suggested that they were migrating cells, expressed IP3 R1 mRNA. However, there was no protein signal from the...
ventricular zone at any age or in the cells migrating radially across the intermediate zone in sagittal, horizontal or coronal sections from E14 to newborn. The protein staining was always observed once the neurons reached the cortical plate.

In the newborn and P3 some neuronal perikarya were stained in all cortical layers (Fig. 5C). By P7-P14 the expression was slightly heavier and extended to all cells (Fig. 5D). The staining appeared in some neuronal processes by P14, and increased by P21, especially in the large neurons. However, it was not until P28 that the levels of expression of layers II and V were markedly increased relative to the other neurons.

Expression of IP₃R1 in the striatum and olfactory bulb

The caudate-putamen was strongly labelled in the adult rat. During development the IP₃R1 was first detected in very few cells and at very low levels at P3. From P10 all the cells were labelled, and increased significantly by P21 (data not shown).

In the olfactory bulb strong expression of IP₃R1 mRNA was observed only in the large mitral cells from early in development, and in some cells of the granular layer. The protein was detected in the same regions but at very low levels from P3. This contrasts with previous studies that report staining in the periglomerular layer and granule cells of the mitral cell layer, with occasional weak staining in the mitral cells (Sharp et al., 1993a). The discrepancy might reflect the use of non-type-specific antibodies that might cross-hybridize with other isoforms. Immunoreactivity was also present in the olfactory cortex. This contains cells that receive direct synaptic input from the olfactory bulb, e.g. olfactory peduncle, olfactory tubercle, entorhinal cortex, insular cortex, perirhinal cortex and cortical areas associated with the amygdala (data not shown).

Transient expression of IP₃R1 in glial cells

While most of the neurons were stained in the adult rat, the white matter was completely devoid of IP₃R1. This was better seen using in situ hybridization (Fig. 6A). However, during development we observed strong labelling in the cell bodies of fibre tracts (Fig. 6B), such as the fimbria from the hippocampus (Fig. 6C) and the corpus callosum at P14 (Fig. 6D). The staining was generalized to the white matter and the levels increased during the next few days, but a marked decline by P28 was evident and by P56 the staining was absent from all white matter.

Expression of IP₃R1 in the spinal cord

At all postnatal ages from P0 to adult, a similar pattern of IP₃R1 mRNA was found in the grey and white matter of the spinal cord (Fig. 7A). A subpopulation of dorsal and ventral horn cells was labelled. The cellular composition was more clearly seen when the same section, labelled with Hoechst nuclear dye, was viewed under UV optics to display the nuclei. The asterisks in Fig. 7D,E show the same field of the ventral horn under bright-field (Fig. 7D) and UV (Fig. 7E) optics. Most nuclei were intensely fluorescent with the Hoechst dye but their cell bodies were totally unlabelled with the IP₃R1 riboprobe. The asterisk in Fig. 7D corresponds to an unlabelled area marking some fluorescent nuclei in Fig. 7E. The size and position of the few cells stained for IP₃R1 in the ventral horn identified them as motor neurons. The white matter containing oligodendrocytes and astrocytes, but devoid of neuronal cell bodies (Fig. 7F and marked with asterisks in Fig. 7A), was also stained. Preliminary studies using in situ hybridization for IP₃R1 and immunohistochemistry for GFAP as a label for astrocytes in the same section, showed most of the digoxigenin-labelled cells to be GFAP-negative (data not shown). The same pattern of expression was also observed at the protein level (Fig. 7G,H), where staining was apparent in the grey (Fig. 7G) and white matter (Fig. 7H).

During development IP₃R1 mRNA was first detected in the spinal cord as early as E11.5 next to the lumen of the neural tube (Fig. 7B), although the protein was not yet expressed (Fig. 7C). Immunoreactivity was detected very weakly from E12.5, and by E14 IP₃R1 message (Fig. 8A) and protein (Fig. 8B) were highly expressed. In glial cells IP₃R1 was observed very weakly during the first postnatal week, and after P10 a stronger signal was evident (Fig. 7F). The levels of expression increased during the next days and the label remained throughout development.

Expression of IP₃R1 in ganglia

We analyzed the neurons from the PNS, and found that IP₃R1 was also expressed in the dorsal root ganglia (DRG) (Fig. 8). During development IP₃R1 message was first detectable in DRG neurons at E12.5 and protein by E14 (Fig. 8B), but we could not detect any signal at E11.5 (Fig. 7B,C). The signal was not generalized to all the DRG neurons when in situ hybridization was performed at E18 (Fig. 8C), suggesting that...
only a subpopulation of DRG neurons expressed IP3 R1. It was also possible to detect staining in other ganglia, such as the trigeminal (Fig. 8D) and sympathetic ganglia (data not shown).

In the adult rat IP3 R1 message was very strong and clear in DRG neurons (Fig. 8E). However, at the protein level, weak immunoreactivity was observed in most regions of the cytoplasm (Fig. 8F,G) and in some neurons a strong signal was associated with the nuclei (Fig. 8G). There were also some unlabelled cells (Fig. 8F,G), counterstained in blue, which from their size and position seem to be Schwann cells and satellite cells.

**DISCUSSION**

It has been known for some time, that calcium has a central role in the regulatory and signalling mechanisms of neurons (Kennedy, 1989). Intracellular Ca2+ is of major importance in the regulation of growth cone motility and acts as a common integrator in the plasticity of neurite outgrowth and synaptogenesis (Kater et al., 1988). For example, the rise of intracellular Ca2+ concentration triggers both the release of neurotransmitters (Kennedy, 1989) and the transcription of immediate-early genes in neurons, such as c-jun and c-fos (Morgan and Curran, 1989).

In this study we have described the localization of IP3 R1 at some points in the development of the rat nervous system using in situ hybridization and immunohistochemistry. Broadly the same pattern of expression was observed at both RNA and protein level, corroborating the specificity of the IP3 R1 probe and antibody we used. In the adult brain, IP3 R1 expression was seen at highest levels in the cerebellum, followed by the CA1 region of the hippocampus, caudate putamen and cerebral cortex, in agreement with previous work in which a non-type specific antibody was used (Nakanishi et al., 1991; Sharp et al., 1993a; Rodrigo et al., 1993), suggesting that type 1 IP3R staining was a major contributor to the labelling observed in previous studies.

**Fig. 3.** Expression of IP3 R1 in developing rat cerebellum. Immunostaining of a sagittal section of E14 (A); E18 (B); P0 (C), showing labelling in the anterior vermis; P3 (D); P7 posterior vermis (E), the arrow indicates stained axons in myelinated fibres; anterior vermis of P7 (F), which differentiates later in development. (G) In situ hybridization and (H) immunostaining of P14; the arrows point to labelled cells in the granular layer. Abbreviations as in Fig. 2. Scale bars: 25 μm (A,B,D); 50 μm (C); 100 μm (E,F); 75 μm (G) and 10 μm (H).
In the cerebellum we observed the expression of IP3 R1 as early as E14 in the rostral part of the cerebellar plate, and on days E17 and E18 we detected labelled cells both in the rostral and posterior region, beneath the germinal layer. These cells were primordial Purkinje cells, as seen by tracing their progress on subsequent days.

Altman (1982) has shown that Purkinje cells originate in the posterior region near the neuroepithelium on day E13 (we have converted the dates reported by Altman to the E0 convention we use) and remain quiescent until E16, after which the neurons migrate to the surface of the dorsal region beneath the germinal layer. Once they reach the surface the cells project their axons and establish some form of cell contact. In our sections we did not see, at any embryonic age, any stained cells in the neuroepithelium or migrating into the surface of the cerebellar plate. This suggests that the labelled cells we observed at E14 were Purkinje cells that have already migrated to their final positions. It is possible that these neurons labelled at E14 correspond to the differentiating large neurons reported by Altman (1982) as those of the locus coeruleus, since their location is similar.

The scattering of Purkinje cells in several rows at birth has also been described by Altman (1972), although in contrast to our results, he describes rows of 6-12 cells, while we only observed rows of 2-4 cells. He also observed that the Purkinje cells closer to the surface appear to be more mature than the others, something we did not observe. It is possible that the Purkinje cells expressing IP3 R1 correspond to the more mature cells which Altman described. This also correlates with the regional differences in the levels of IP3 R1 observed in the newborn rats, which has been associated with differences in the maturation of the Purkinje cells (Altman and Bayer, 1985), and implies that the level of expression increases as the cell differentiates.

When the Purkinje cells had migrated to their final position
in the cerebellar cortex all the cells were labelled, forming several rows of cells. At this age (P3) very few synaptic contacts have formed and the axons have a ‘tubular’ organization (Altman, 1972). Ca2+ responses have also been determined at P3 (Llinás and Sugimori, 1979). As the Purkinje cells differentiate in a caudal to rostral gradient by P7, the IP3R1 starts to be expressed throughout the axons, and at this stage, the labelling extends into the white matter. At this age asymmetrical synapses in the somata of the Purkinje cells start to proliferate, but it is not until P14 that symmetric synapses and the dendritic spines are formed, consistent with the continuous increase of label.

We can conclude firstly, that by E14 some of the Purkinje cells have reached the surface and express IP3R1, earlier than previously reported, and start forming a layer not only from the posterior part but also from the anterior part of the dorsal region. Secondly, the Purkinje cells express IP3R1 after the cells have migrated to their final position, when they start projecting their axons. Thirdly, the level of IP3R1s increases as the Purkinje cells differentiate and fourthly, the expression in the axons is correlated with the appearance of synapses in the somata of the Purkinje cells.

In previous studies it was shown that the earliest appearance of IP3R1 in Purkinje cells was E17 in mouse (Nakanishi et al., 1991) and P3 in rat (Maeda et al., 1989), but we detected the receptor as early as E14. The discrepancy may be due to methodological differences.

In the cerebellum the axons of the Purkinje cells represent the only output system, with very long axons and the most extensive dendritic arborization in the brain. The Purkinje cells receive an enormous number of synapses, which is reflected in the complexity of their integrative properties (Llinás and Walton, 1990). Probably the very high levels of expression of IP3R1 from very early in development in the cerebellum are associated with more than one mechanism being involved in the regulation of information processing from various sources, in response to environmental changes.

**Hippocampus**

In the adult hippocampus, the levels of IP3R1 in CA1 pyramidal cells were higher than in other neurons, which is in agreement with other studies (Nakanishi et al., 1991; Sharp et al., 1993a). However, the distribution in the hippocampus is different from the other Ca2+ receptor channel, ryanodine, which has been reported to be enriched in the dentate gyrus (Lai et al., 1992) or in the CA2-CA3 (Nakanishi et al., 1992) neurons. It is possible that the distinct patterns of distribution of IP3 and ryanodine receptors reflect physiological differences between both Ca2+ receptor channels.

During the postnatal development of the hippocampus, the stratum radiatum in the CA1 region progressively grows relatively more than the stratum molecular, this change being evident by P12 in rat (Zimmer and Haug, 1978), and implies that the CA1 dendrites grow not only at their tips but also intralaminarly. Some studies have also suggested that dendritic growth might be induced by axon-dendritic synapses, which increase significantly in rats during the second and third postnatal week (Zimmer, 1978). This is the period in which the expression of IP3R1 in CA1 increased relative to the levels of the other neurons. There is also an increase in the number of spines in CA1 pyramidal neurons during this period of development. By P10-20 in rats there is a fourfold increase in the spine dendrites (Zimmer, 1978). IP3R1s have been reported to be expressed in the spines of Purkinje cells (Walton et al., 1991), so they might well be present in the spines of the CA1 pyramidal cells. The local increase in the intracellular concentration of calcium in the postsynaptic spines in the adult hippocampus has been shown to trigger long-term potentiation (Malenka et al., 1989).

The delay in the accumulation of IP3R1 in CA1 is also comparable to the appearance of the laminar pattern observed in Timm sulphide silver (Zimmer and Haug, 1978) and Golgi (Minkwitz and Holz, 1975) stained material. In conclusion, the increasing levels of IP3R1 in CA1 pyramidal neurons and dendrites, after the second postnatal week, coincide with the extensive dendritic branching and elongation of the apical pyramidal cell dendrites, the establishment of mature synapses and the increasing formation of spines within the layer during this period.

In the adult granule neurons of the dentate gyrus, only a proportion of the cells expressed a low level of IP3R1 and labelling was not observed in any of their processes. This result contrasts
with previous work in which staining was reported in the dentate gyrus (Sharp et al., 1993a; Rodrigo et al., 1993), maybe because these studies were carried out with non-type-specific antibodies. It has been known for some time that granule neurons continue to be generated not only at early postnatal times, but also in mature adults (Kaplan and Hinds, 1977). We observed that during development the more mature cells express more IP3 R1. As the cells gradually accumulate in the granular layer, since there is no substantial turnover of this neuronal population (Crespo et al., 1986), the cells start to express IP3 R1 and the proportion of labelled cells increases. However, the cells that continue to be generated, facing the hilus, are negative even in the adult rat. In conclusion, IP3 R1 begins to be expressed in the granular neurons of the dentate gyrus as the cells start to differentiate.

Cerebral cortex

In the adult neocortical plate, IP3R1 was expressed in all the neuronal layers, but higher levels of immunostaining were observed in the non-pyramidal and pyramidal layers, II and V respectively, in agreement with Sharp et al. (1993a). During development neocortical neurons arise from the neuroepithelium of the ventricular zone and migrate across the intermediate zone, following the processes of radial glia. The neurons accumulate in the preplate, which later generates the cortical plate and eventually all the definite cellular layers (O’Leary and Koester, 1993). Most neurons do not begin axogenesis until their migration is substantially completed and they have reached their final laminar destination (De Carlos and O’Leary, 1992; Shoukimas and Hinds, 1978). In our experiments IP3R1 mRNA was observed in the ventricular layer and the neocortical plate, but we could not identify any labelled cells migrating across the intermediate zone, and IP3R1 protein was not observed until the neurons arrived in the cortical plate. The lack of IP3R1 in the migrating neurons and its expression in the neocortical plate suggest that IP3R1 expression might be triggered by the onset of axogenesis.

The levels of IP3R1 were also very low in embryos and in the first days of life, but a dramatic increase was seen between P10 and P14. Levels continued to increase until P21 after which the levels remained relatively unchanged. During this period the density of type II synapses increases approximately twofold and at P14 they are distributed in all layers of the cortex (Aghajanian and Bloom, 1967; Blue and Parnavelas, 1987). Also, an increase in spine density is first observed in dendrites of between P12 to P20 (Parnavelas and Globus, 1976). In conclusion, the expression of IP3R1 in the neocortical neurons is probably triggered by the onset of axogenesis,
Expression of IP$_3$ R during axiogenesis and the increasing level of receptors in the cortical neurons during the second and third postnatal week coincides with the establishment of type II synapses and the increasing formation of spines during this period of development.

**Spinal cord**

A different pattern was seen in the spinal cord. While in the adult brain IP$_3$R1s were expressed only in neurons, in the spinal cord they were observed in both neurons and glial cells. Expression of IP$_3$R has been reported in the dorsal and ventral horn of the adult spinal cord (Sharp et al., 1993a), but not in the white matter. During development a delay between the appearance of IP$_3$R1 mRNA and protein of at least 24 hours was observed in the spinal cord, suggesting that an additional signal is required for protein expression. Altman and Bayer (1984) have shown that the onset of cell differentiation and axogenesis in the spinal cord is evident between day E12-E14, after the proliferative phase in the neuroepithelium. This correlates with the period in which IP$_3$R1 starts to be expressed in the spinal cord.

**Gliaal cells**

Gliaal cells did not express IP$_3$R1 in the brain, except for a transient period in developing fibre tracts. Most astrocytes are generated during the first week of postnatal development in the rat optic nerve, while the majority of oligodendrocytes are generated during the second postnatal week, at the time when the tracts are rapidly being myelinated (Skoff, 1990; Skoff and Knapp, 1991). Several studies have also indicated a strong correlation in time between the onset of myelination and the rise in glial population in different parts of the white matter (Matthews and Duncan, 1971). In our results IP$_3$R1 was first detected in the fibre tracts of the rat brain during P10-P14, suggesting that IP$_3$R1 is most likely to be expressed in oligodendrocytes during the period of proliferation and the onset of myelination. A downregulation in the expression of IP$_3$R1 was observed after a few days, which was substantially completed by P28 and finished by P56. A similar transient expression has been observed with urokinase-type plasminogen activator (uPA) (Dent et al., 1993) and also a transient activity of oxidative enzymes such as NAD-diaphorase (Schönbach et al., 1968). During myelin formation and oligodendrocyte proliferation, uPA mRNA expression was observed and the activity of NAD-diaphorase increased; but as the myelin action process slowed down, the enzymatic activity dropped to a very low level and uPA expression was not detected.

Gliaal cells were also labelled in the spinal cord, and this was first detected during the second week of development, followed by a rise in the levels of IP$_3$R1 expression during the next few days; but in contrast with the brain, gliaal cells remained labelled throughout development. Some studies in the spinal cord have reported that oligodendrocytes constitute 85% of the cells in the tracts during the first weeks of development and that a rise in the oligodendrocyte population is observed during myelin formation (Matthews and Duncan, 1971). These observations, and our preliminary results in which GFAP astrocytes were not positively labelled for IP$_3$R1, suggest that the gliaal cells labelled in the spinal cord are oligodendrocytes. Also, the time when IP$_3$R1 was detected in the oligodendrocytes coincides with the onset of myelination.
In conclusion, IP$_3$R1 is probably expressed in the oligodendrocytes in both brain and spinal cord during the period of myelination. However, in the brain IP$_3$Rs are expressed only for a transient period of development and this might reflect the presence of other types of IP$_3$R in oligodendrocytes, while in the spinal cord they are expressed throughout development and are still detectable in the adult spinal cord. This suggests that IP$_3$R1 might play another role in the oligodendrocytes of the spinal cord as myelination process slows down in development. One reason IP$_3$Rs might be maintained in spinal cord oligodendrocytes throughout life is that the spinal cord contains large number of very long axons, some of which are myelinated by rows of single oligodendrocytes in a 1:1 relationship with the axons, like Schwann cells (Remahl and Hildebrand, 1990). The requirements for maintaining a large myelin sheath round a single axon are likely to be greater than for maintenance of several smaller sheaths by one oligodendrocyte, as seen for instance in the optic nerve. Intracellular Ca$^{2+}$ might be important in this event.

Peripheral nervous system

The expression of IP$_3$R1 was not restricted to the central nervous system, but was also observed in the peripheral nervous system. We report here for the first time its expression in the dorsal root ganglia and also in trigeminal and sympathetic ganglia. During development this expression appears to coincide with the differentiation of neurons into a more bipolar shape and the onset of axogenesis (Altman and Bayer, 1984).

The panorama that emerges from this study is that type I IP$_3$R might be involved in more than one event, both during development and among different types of cell. It is likely that the absence of IP$_3$R1 in most glial cells and other neurons reflects the expression of other isoforms in these cells, with different patterns of distribution and different physiological roles between them.

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