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Multiple Types of Ryanodine Receptor/Ca^{2+} Release Channels Are Differentially Expressed in Rabbit Brain

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The neuronal Ca^{2+} signal is induced by a rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and is thought to be important for higher brain function. Dynamic changes in $[Ca^{2+}]_i$ are affected by the spatial distributions of various Ca^{2+}-increasing molecules (channels and receptors). The ryanodine receptor (RyR) is an intracellular channel through which Ca^{2+} is released from intracellular stores. To define the contribution of neuronal Ca^{2+} signaling via the RyR channel, we examined RyR type-specific gene expression in rabbit brain by *in situ* hybridization histochemistry. The neuronal RyR was composed of three distinct types, two types dominant in skeletal (sRyR) and cardiac (cRyR) muscle, respectively, and a novel brain type (bRyR). sRyR was distinguished by its high level of expression in cerebellar Purkinje cells. cRyR was predominantly expressed throughout nearly the entire brain, and was characterized by its markedly high level of expression in the olfactory nerve layer, layer VI of the cerebral cortex, the dentate gyrus, cerebellar granule cells, the motor trigeminal nucleus, and the facial nucleus. bRyR expression was the least widely distributed throughout the brain, and was high in the hippocampal CA1 pyramidal layer, caudate, putamen, and dorsal thalamus. This investigation demonstrates that the heterogeneous distribution of neuronal RyRs may be implicated in distinct Ca^{2+}-associated brain functions. Moreover, it should be noted that cRyR, a typical CICR channel, is distributed widely throughout the brain, suggesting that in a variety of cell types, the amplification of neuronal Ca^{2+} signals is functionally accompanied by a rise in $[Ca^{2+}]_i$, such as Ca^{2+} influx stimulated by neuronal activity. This widespread distribution of the neuronal RyR family indicates that Ca^{2+} signals via the intracellular stores should be considered in studies of neuronal Ca^{2+} dynamics.

[Key words: ryanodine receptor, Ca^{2+}-induced Ca^{2+} release, intracellular free Ca^{2+}, neuronal Ca^{2+} signal, inositol 1,4,5-trisphosphate receptor, in situ *hybridization]*

Dynamic changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) play a crucial role in numerous neuronal functions, including the movement of the growth cone, release of neurotransmitters, initiation and maintenance of long-lasting changes in synaptic transmission efficacy such as long-term potentiation (LTP) in the hippocampus and long-term depression (LTD) in the cerebellum, the transcription of immediate-early genes, and neurotoxicity (Kennedy, 1989), in addition to the ubiquitous so-called *housekeeping* functions. Neuronal $[Ca^{2+}]_i$ is well regulated by homeostatic mechanisms (Ca^{2+} transporting and buffering systems) that maintain its resting cytosolic concentration ($\approx 10^{-7}$ M) (Blaustein, 1988). In order to act as signals, $[Ca^{2+}]_i$ must increase at least severalfold. With neuronal activity, a rise in the $[Ca^{2+}]_i$ is triggered by two pathways: (1) Ca^{2+} influx across the plasma membrane by voltage-operated Ca^{2+} channels (Tsien et al., 1989) and Ca^{2+}-permeable ligand-gated ion channels (Barnard, 1992), and (2) mobilization of Ca^{2+} from internal stores by ryanodine receptors (RyRs) (Endo, 1977; Fleischer and Inui, 1989) and inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Berridge and Irvine, 1989; Furuichi et al., 1992). These different Ca^{2+}-increasing systems show the distinct spatiotemporal dynamics of $[Ca^{2+}]_i$ transients within a cell, which are thought to be important for the functional diversity of Ca^{2+} signaling. The spatial dynamics of Ca^{2+} signaling are crucially affected by the heterogeneous spatial distribution of a variety of plasmalemmal and organellar channel types within the cell. This local $[Ca^{2+}]_i$ elevation is thought to be a possible mechanism underlying the functional specialization of neuronal Ca^{2+} signals, and appears to decide a range of target proteins, activated or inactivated, many of which generally have their characteristic subcellular localizations and some of which are known to show dependence on a narrow $[Ca^{2+}]_i$ range. Thus, to understand the Ca^{2+} signaling function in a particular neuron, it is important to know what kinds of Ca^{2+}-increasing molecules are expressed in the cell.

The organellar Ca^{2+} release channels IP_3R and RyR are responsible for distinct Ca^{2+} mobilization systems from internal stores, but the physiological roles of Ca^{2+} signaling via these

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channels in the brain are still unclear, in contrast to the plasmalemmal Ca^{2+} channels. The IP_3R channel releases Ca^{2+} in response to binding of the second messenger IP_3 produced by the phosphoinositide signal transduction cascade, for example, the successive activation of a plasma membrane receptor, G protein, and then phospholipase C (Berridge and Irvine, 1989). Although phosphoinositide turnover is considered to be involved in crucial parts of higher brain function, such as LTP in the hippocampus (Murphy and Miller, 1988; Lester and Jahr, 1990) and LTD in the cerebellum (Ito and Karachot, 1990; Linden et al., 1991), there is no strong evidence with respect to the substantial role of IP_3-induced Ca^{2+} release (IICR) in brain function. The functional role of RyR-mediated Ca^{2+} release in the dynamic changes in neuronal $[Ca^{2+}]_i$ is even more unclear. Release of Ca^{2+} from the intracellular stores of neurons by caffeine, analogous to muscle RyR function, has been reported in peripheral (Kuba and Nishi, 1976; Kuba, 1980; Smith et al., 1983; Neering and McBurney, 1984; Thayer et al., 1988a,b) and central (Martinez-Serrano and Satrustegui, 1989; Murphy and Miller, 1989; Glaum et al., 1990) neurons. Ca^{2+} waves and oscillations in frog sympathetic neurons (Kuba and Takeshita, 1981; Smith et al., 1983; Lipscombe et al., 1988) appear to be due partly to Ca^{2+}-induced Ca^{2+} release (CICR), which would be compatible with cardiac RyR function. Thus, caffeine-sensitive and Ca^{2+}-induced Ca^{2+} release in neurons may be attributable to neuronal RyR function. The existence of Ca^{2+} stores in the dendritic spines of the dentate molecular layer was demonstrated using a pyroantimonate precipitation technique (Fifková et al., 1983). Caffeine abolishes posttetanic potentiation in rat hippocampal synapses (Lee et al., 1987), and dantrolene and thapsigargin, drugs that inhibit the CICR activity of RyR and deplete intracellular Ca^{2+} pools, respectively, can inhibit the induction of LTP in the CA1 region of rat hippocampal slices (Obenaus et al., 1989; Harvey and Collingridge, 1992). Recently, upon stimulation of associative-commissural inputs, the sustained $[Ca^{2+}]_i$ elevation in the spine heads of hippocampal CA3 pyramidal cell dendrites was recorded (Müller and Connor, 1991; see also Regehr and Tank, 1992), which has been considered to be due to CICR (regenerative $[Ca^{2+}]_i$ transients). These results suggest that these neuronal RyRs are probably important for higher brain function (Miller, 1992; Bliss and Collingridge, 1993). In cerebellum, it was also shown that cultured cerebellar neurons definitely have caffeine-induced Ca^{2+} release activity (Brorson et al., 1991; Yuzaki and Mikoshiba, 1992).

Through molecular cloning, we now know that mammals have at least three distinct types of RyR: two muscle RyRs, the skeletal type (sRyR) (Takeshima et al., 1989) and the cardiac type (cRyR) (Nakai et al., 1990; Otsu et al., 1990); and a novel brain type RyR (bRyR) (Hakamata et al., 1992). The type names are based on dominant tissue distribution (sRyR and cRyR) or the origin of molecular cloning (bRyR). sRyR and cRyR are involved in excitation–contraction (E-C) coupling in skeletal and cardiac muscle, respectively. These muscle RyRs have been immunohistochemically localized in chicken (Ellisman et al., 1990; Ouyang et al., 1991; Walton et al., 1991) and fish (Zupanc et al., 1992) brain using the sRyR probe, and in rodent brain (Kuwashima et al., 1992; Lai et al., 1992; Nakanishi et al., 1992; Sharp et al., 1993) using the sRyR and cRyR probes and the nonspecific probe. sRyR was exclusively localized in cerebellar Purkinje cells (Ellisman et al., 1990; Walton et al., 1991; Kuwashima et al., 1992), while cRyR was shown to be the predominant type in brain (McPherson et al., 1991; Kuwashima et al., 1992; Lai et al., 1992; Nakanishi et al., 1992). Widespread distribution of RyR was shown by Western blotting (Kuwashima et al., 1992) and immunohistochemical (Nakanishi et al., 1992) studies mainly using the probe nonspecific for all RyR

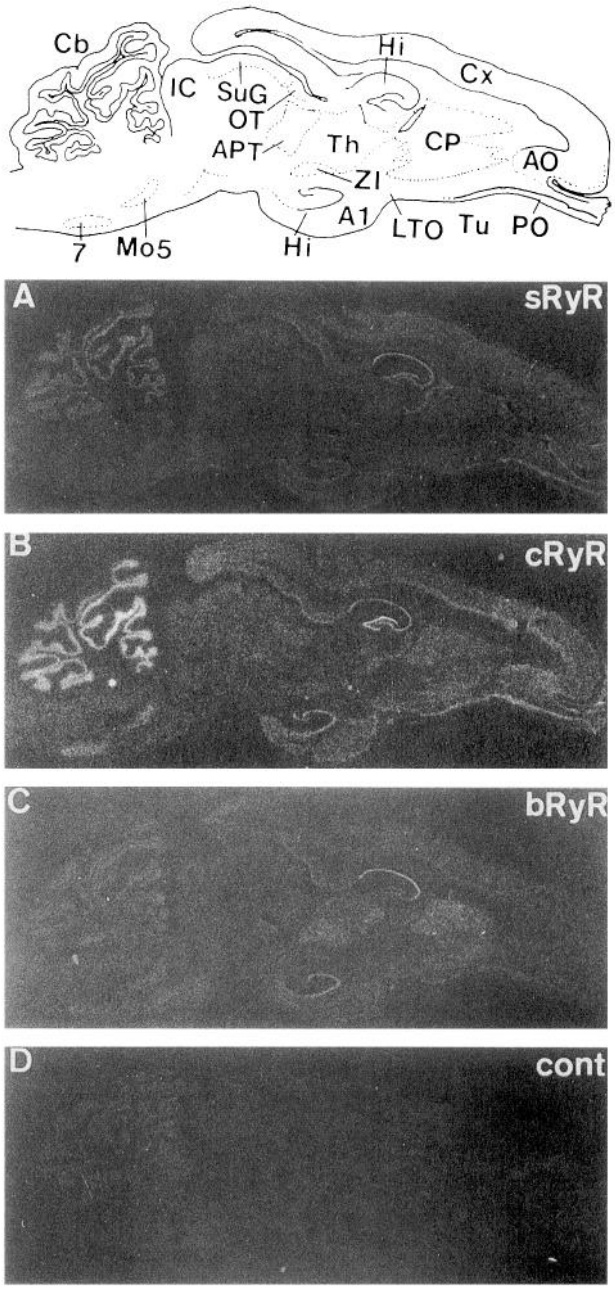

Figure 1. Heterogeneous distribution of mRNAs of the neuronal RyR family in rabbit brain: film autoradiograms of parasagittal sections hybridized with the ^{35}S-labeled antisense riboprobes for sRyR (*A*), cRyR (*B*), bRyR (*C*), and sense riboprobe for bRyR (*cont, D*). Films were exposed for 18 d (sRyR, *A*), 5 d (cRyR, *B*), or 14 d (bRyR, *C*; control, *D*). Anatomy is depicted schematically at the *top*. *AO*, anterior olfactory cortex; *PO*, primary olfactory cortex; *Tu*, olfactory tubercle; *LTO*, lateral olfactory tract; *Cx*, cerebral cortex; *CP*, caudate and putamen; *Hi*, hippocampus; *Th*, thalamus; *ZI*, zona incerta; *A1*, anterior cortical and medial amygdaloid nuclei, and amygdalohippocampal area; *OT*, nucleus of the optic tract; *APT*, anterior pretectal area; *SuG*, superficial gray layer of the superior colliculus; *IC*, inferior colliculus; *Cb*, cerebellum; *Mo5*, motor trigeminal nucleus; *7*, facial nucleus.

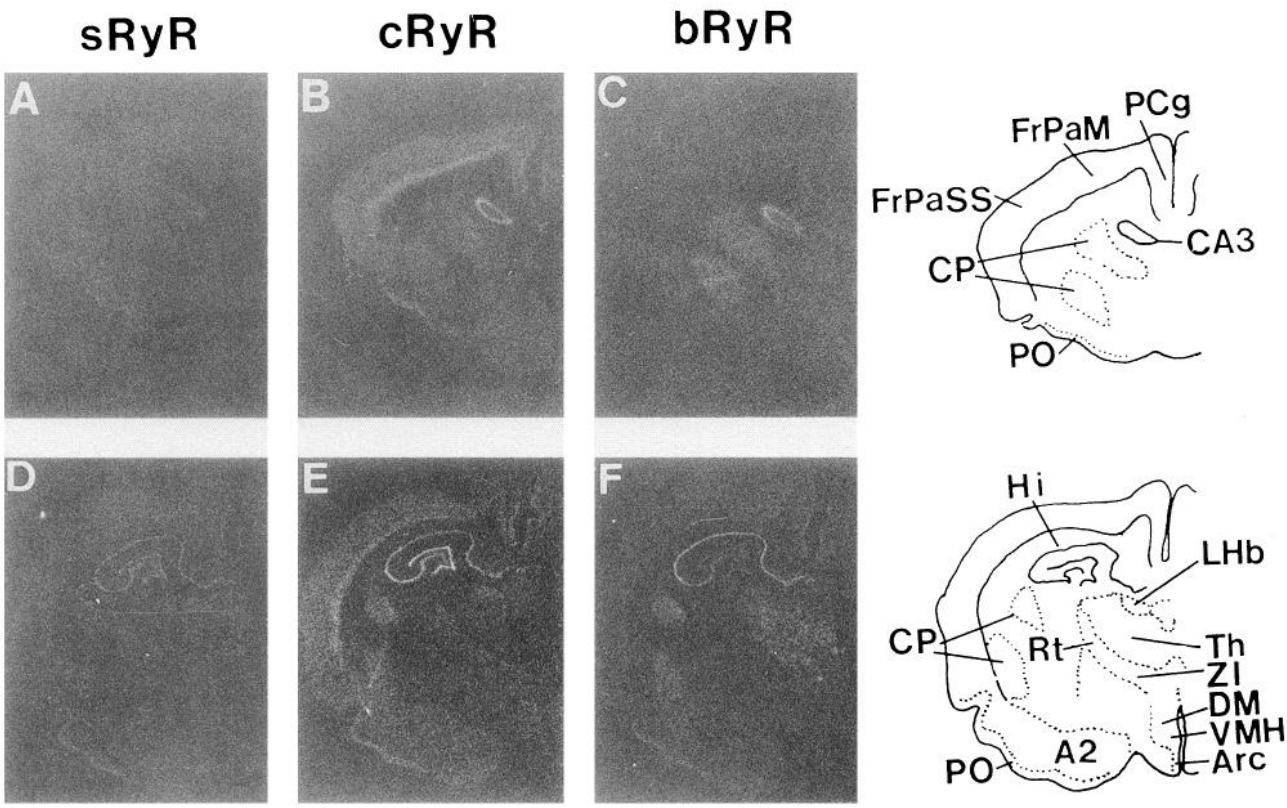

Figure 2. Heterogeneous distribution of mRNAs of the neuronal RyR family in rabbit forebrain: film autoradiograms of coronal sections hybridized with the ^{35}S-labeled antisense riboprobes for sRyR (*A, D*), cRyR (*B, E*), and bRyR (*C, F*). Films were treated as described for Figure 1. Anatomy is depicted schematically at the *right*. *PCg,* posterior cingulate cortex; *FrPaM,* frontoparietal cortex motor area; *FrPaSS,* frontoparietal cortex somatosensory area; *CA3,* CA3 pyramidal cell layer of the hippocampus; *CP,* caudate and putamen; *PO,* primary olfactory cortex; *Hi,* hippocampus; *LHb,* lateral habenular nucleus; *Th,* thalamus (posterior thalamic nuclear group, paracentral, central medial, centrolateral, mediodorsal, paraventricular, ventrolateral, ventromedial, and ventroposterior thalamic nuclei); *Rt,* reticular thalamic nucleus; *ZI,* zona incerta; *DM,* dorsomedial hypothalamic nucleus; *VMH,* ventromedial hypothalamic nucleus; *Arc,* arcuate hypothalamic nucleus; *A2,* central, lateral, basolateral, medial, anterior cortical, and posterolateral cortical amygdaloid nuclei, and amygdalohippocampal area.

types. However, now we know that none of the RyR probes used in the previous immunological studies is capable of distinguishing the bRyR type specifically from either sRyR or cRyR because of the cross-reactivity of the probes used. Therefore, it remained unclear which type of RyR family was localized in a particular neuron. Yoshida et al. (1992) reported that more than 85% of ^{3}H-ryanodine binding sites from rat brain were immunoprecipitated by the anti-cRyR monoclonal antibody, and therefore claimed no cross-reactivity of the antibody with sRyR. Smith and Nahorski (1993), on the other hand, claimed that

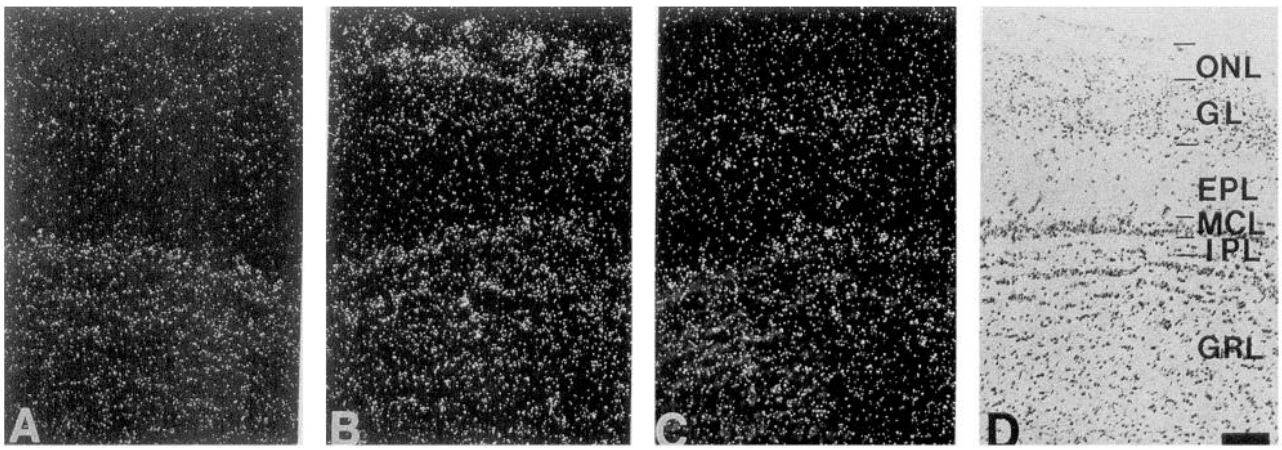

Figure 3. Differential localization of neuronal RyR mRNAs in the rabbit olfactory bulb: dark-field observations of coronal sections of olfactory bulbs hybridized with ^{35}S-labeled sRyR (*A*), cRyR (*B*), and bRyR (*C*) probes and dipped into NTB2 emulsion followed by exposure for 12 d (*B*) or for 38 d (*A* and *C*). *D,* Bright-field observation of serial adjacent sections stained with hematoxylin and eosin. *ONL,* olfactory nerve layer; *GL,* glomerular layer; *EPL,* external plexiform layer; *MCL,* mitral cell layer; *IPL,* internal plexiform layer; *GRL,* granule cell layer. Scale bar, 100 μm.

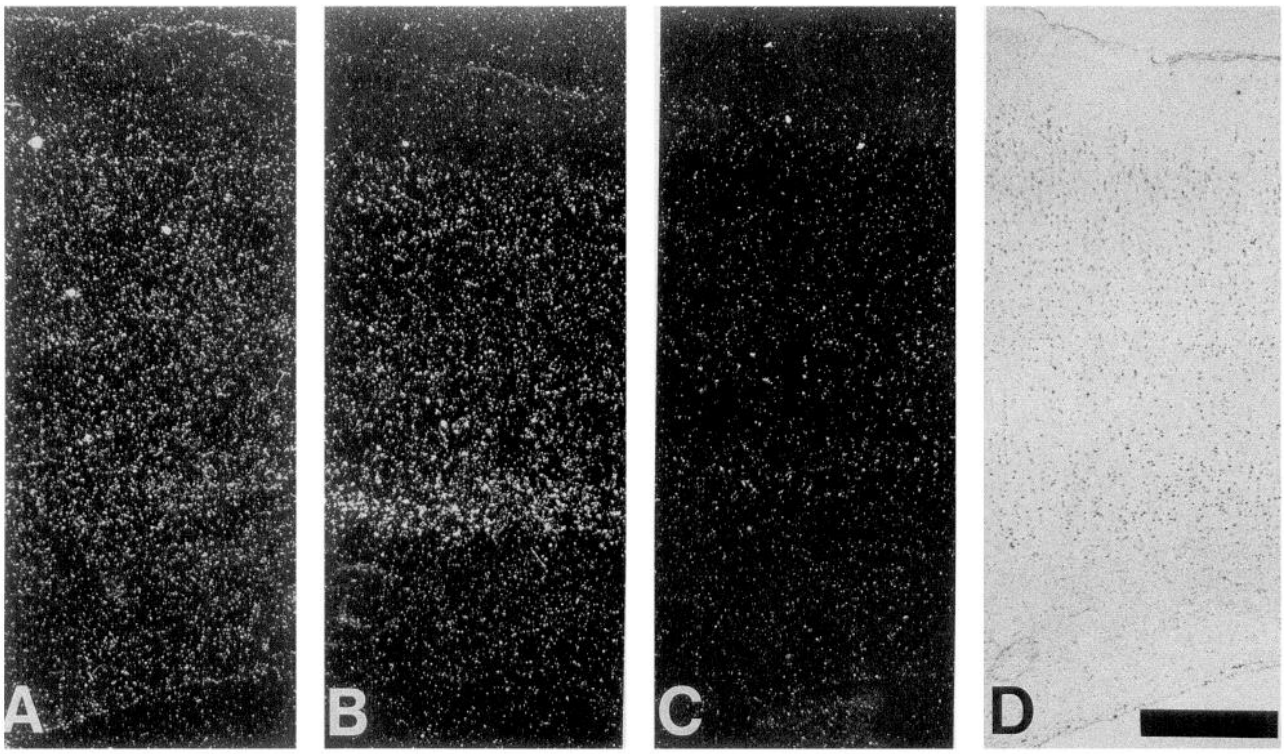

Figure 4. Differential localization of neuronal RyR mRNAs in rabbit cerebral cortex: dark-field observations of coronal sections of the frontoparietal cortex hybridized with ^{35}S-labeled sRyR (*A*), cRyR (*B*), and bRyR (*C*) probes. *D*, Bright-field observation of serial adjacent sections stained with hematoxylin and eosin. Scale bar, 500 μm.

the ^{3}H-ryanodine binding properties of rat cortical membranes were more similar to those of sRyR than cRyR. Neither group considered the existence of bRyR in the brain. Therefore, the RyR types and the predominant type expressed in brain should be defined.

In this study, we localized each member of the RyR family in the rabbit brain in detail by *in situ* hybridization using RyR type-specific probes. The rabbit neuronal RyR family was composed of all of these RyRs. The present study has more clearly demonstrated the heterogeneous distribution of each neuronal type, and is the first to localize bRyR mRNA in particular neurons. Upon examining the expression levels of all RyR-type mRNAs, we confirmed that cRyR overwhelmingly predominates throughout most of the rabbit brain. In comparison with sRyR and cRyR, the expression of bRyR mRNA is highly characteristic and restricted, with the highest level in hippocampal CA1 pyramidal cells followed by the caudate, putamen, accumbens nucleus, and dorsal thalamus. The heterogeneous distribution of functionally distinct RyR types may be involved in the differential roles in neuronal Ca^{2+} signaling.

Materials and Methods

Cryosection preparation. Rabbits (std: JW/CSK, 13 week old) were obtained from Nippon SLC Co. (Shizuoka, Japan). After being anesthetized with sodium pentobarbital (0.5 mg/kg of body weight), the rabbits were intracardially perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (PFA fixer). After perfusion, brains were dissected, postfixed in PFA fixer for 1.5 hr on ice, and immersed in 25% sucrose in PBS for 16 hr at 4°C for cryoprotection. The brains were frozen and sectioned at 12 μm.

Riboprobe preparation. pBluescript plasmids harboring the cDNA fragments of sRyR, cRyR, and bRyR, which diverged adequately from each other, were used: the *Eco*RI-*Eco*RI fragment of pRR229 (1443–4431 nucleotide positions; Takeshima et al., 1989), the *Eco*RI-*Sac*I fragment of pHRR12 (11,527–13,623; Nakai et al., 1990), and the *Eco*RI-*Eco*RI fragment of pBRR71 (11,834–13,353; Hakamata et al., 1992), respectively. For the IP_3R1 probe, the *Eco*RI-*Nae*I cDNA fragment (7986–8569 nucleotide positions) cloned in pBluescript plasmid (Furuichi et al., 1989, 1993) was used. Antisense and sense RNAs from these cDNA fragments were synthesized by *in vitro* transcription using T3 and T7 RNA polymerase (Promega) and α-^{35}S-UTP (Amersham).

In situ *hybridization.* *In situ* hybridization was performed as described previously (Furuichi et al., 1993). After hybridization, the samples were exposed to Hyper β-max film (Amersham) for 5 d for the cRyR probe, 14 d for the bRyR probe, and 18 d for the sRyR probe. Some of the samples were dipped in NTB-2 emulsion (Kodak), exposed for 12 d for the cRyR probe and 38 d for the sRyR and bRyR probes at 4°C, and then developed. The developed samples were lightly stained with 0.1% cresyl violet.

Results

mRNAs of the RyR-channel family showed differential distributions in the rabbit brain, although the hybridization intensities with the antisense probes of these receptor types varied considerably (Figs. 1, 2; note the exposure times, see the Fig. 1 caption). cRyR was the most common type of neuronal RyR in almost all regions (Fig. 1*B*). The distribution and approximate expression levels are summarized in Table 1. No significant hybridization signals were observed with the sense probes (for examples, see Fig. 1*D*).

Olfactory region. The mRNAs of the RyR family showed heterogeneous distribution in the olfactory bulb (Fig. 3). cRyR mRNA (Fig. 3*B*) was found at a high density in the olfactory nerve layer, which consists of the axons of olfactory receptor neurons, olfactory nerve Schwann cells which ensheath the axons, and astrocyte subtypes which are morphologically divergent from others and may be associated with the axons (Bailey and

Table 1. Heterogeneous distribution of the ryanodine receptor family

	sRyR	cRyR	bRyR
Olfactory regions			
Olfactory bulb			
Olfactory nerve layer	−/+	+++	−/+
Glomerular layer	−/+	++	++
External plexiform layer	+	+	+
Mitral cell layer	++	++	++
Granule cell layer	+	++	+
Anterior olfactory nu.	+	+++	+
Primary olfactory cortex	++	++++	++
Olfactory tubercles	+	+++	+
Lateral olfactory nu.	+	++	+
Cerebral cortex			
Frontal cortex	+/++	+++	+
Frontoparietal cortex, motor area	+/++	+++	+/++
Frontoparietal cortex, somatosensory area	+/++	+++	+/++
Striate cortex	+	++	+
Anterior cingulate cortex	++	++	++
Posterior cingulate cortex	++	++	++
Agranular region frontopariental cortex, motor area	++	++	++
Retrosplenial cortex	+	++	+
Hippocampal formation			
Entorhinal region	+	+	+
Subiculum	+	+	+
CA1 pyramidal cell layer	+++	+++	++++
CA2 pyramidal cell layer	++	+++	++
CA3 pyramidal cell layer	++	+++	+
CA4 pyramidal cell layer	+	+++	+
Dentate gyrus	++	++++	+
Basal ganglia			
Caudate	+	++	+++
Putamen	+	++	+++
Accumbens nu.	−/+	++	++
Substantia nigra, reticular	−/+	+	−/+
Thalamus			
Lateral habenula nu.	+	++	−/+
Reticular th. nu.	−/+	++	+
Laterodorsal th. nu.	−/+	+	++
Lateral posterior th. nu.	−/+	+	++
Posterior th. nuclear group	−/+	+	++
Paracentral th. nu.	−/+	+	++
Central medial th. nu.	−/+	+	++
Centrolateral th. nu.	−/+	+	++
Mediodorsal th. nu.	−/+	+	++
Paraventricular th. nu.	−/+	+	++
Anteroventral th. nu.	+	+	+++
Ventroposterior th. nu.	+	+	+++
Ventroposterior th. nu. medial	+	+	+++
Ventroposterior th. nu. lateral	+	+	+++
Ventrolateral th. nu.	+	+	+++
Ventromedial th. nu.	+	+	+++
Medial geniculate nu.	+	+	+++
Zona incerta	−/+	++	−/+
Anterior pretectal area	+	++	+
Hypothalamus	+	+	+
Amygdala	+	+	+

Table 1. Continued

	sRyR	cRyR	bRyR
Cerebellum			
Molecular layer	−/+	+	−/+
Purkinje cell layer	++++	++/+++	−/+
Granule cell layer	−/+	+++	−/+
Cerebellar nu.	−/+	++	++
Brainstem			
Nu. optic tract	−/+	++	+
Superificial gray layer of superior colliculus	+	++	+
Inferior colliculus	−/+	++	+
Dorsal nu. lateral lemniscus	−/+	++	+
Ventral nu. lateral lemniscus	−/+	++	+
Dorsal cochlear nu.	−/+	++	+
External cuneate nu.	−/+	++	+
Lateral superior olive	−/+	++	+
Motor trigeminal nu.	+	+++	++
Facial nu.	+	+++	++
Lateral reticular nu.	−/+	++	+
Nu. spinal tract trigeminal nerve	+	++	++

th., thalamic; nu., nucleus. Approximate expression levels of each RyR-type mRNA: ++++, very high; +++, high; ++, medium; +, low; −, not significant. These expression levels were not equilibrated among RyR types.

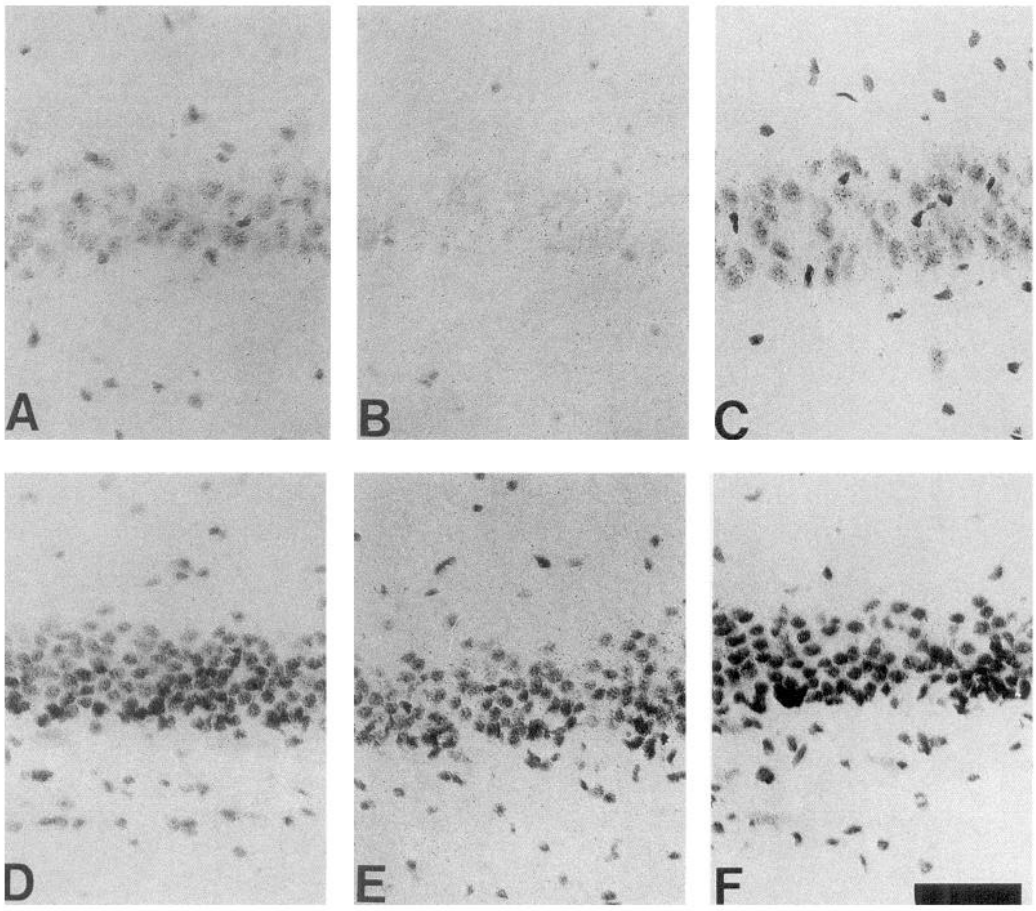

Figure 5. Differential localization of neuronal RyR mRNAs in rabbit hippocampus: bright-field observations of coronal sections of the hippocampus hybridized with ^{35}S-labeled sRyR (*A, D*), cRyR (*B, E*), and bRyR (*C, F*) probes. *A–C* are CA1 pyramidal cell layers, and *D–F* are dentate granular cell layers. Silver grains are seen in and around cell bodies counterstained with cresyl violet. Note: for an easy detection of grains located in dentate granular cells, the sections hybridized with the cRyR probe (*B, E*) are more lightly stained with cresyl violet than the others. Scale bar, 50 μm.

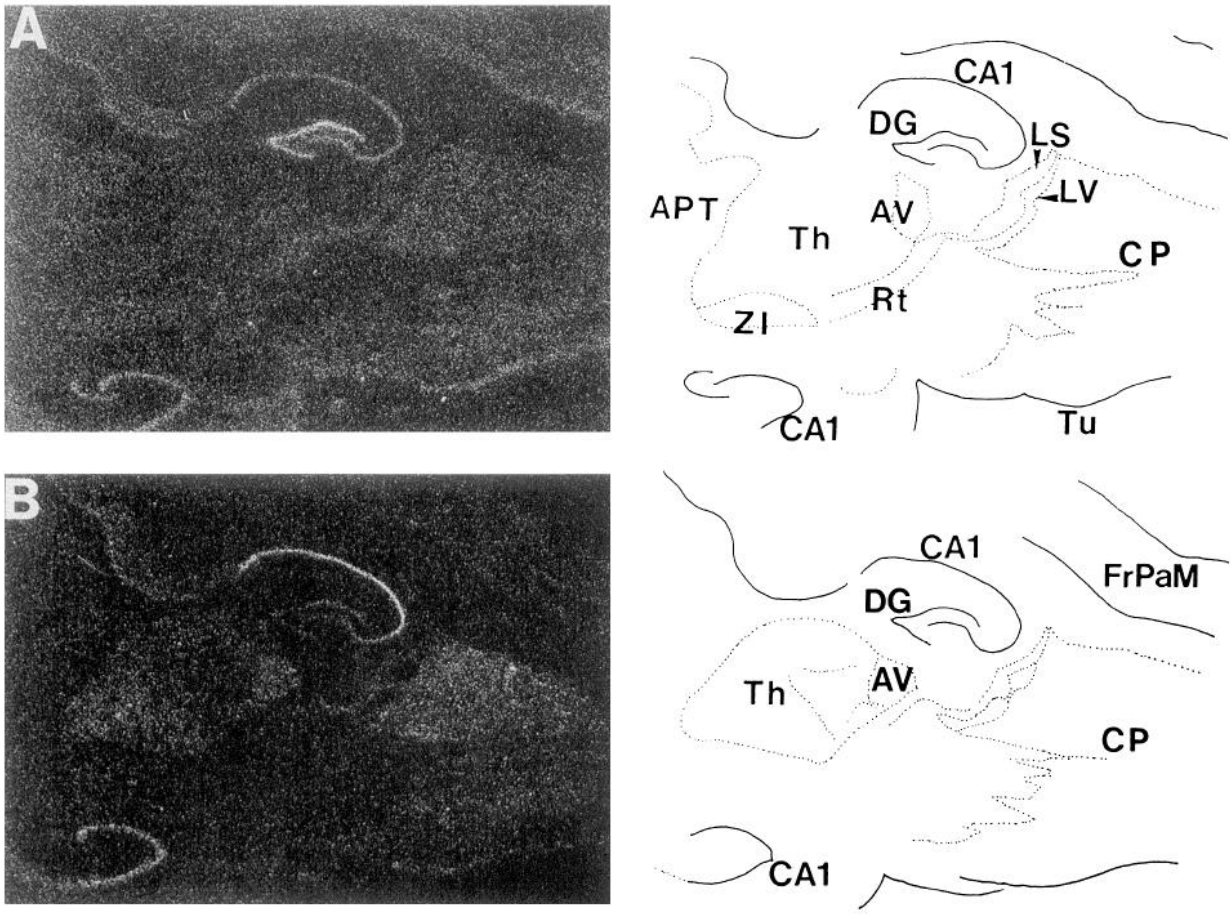

Figure 6. Differential localization of cRyR and bRyR mRNAs in rabbit thalamus: film autoradiograms of parasagittal sections hybridized with ^{35}S-labeled cRyR (*A*) and bRyR (*B*) probes (higher magnification of the region around the thalamus in Fig. 1). Anatomy is depicted schematically at the *right*. *CA1*, hippocampal CA1 pyramidal cell layer; *DG*, dentate gyrus; *LS*, lateral septal nucleus; *LV*, lateral ventricle; *CP*, caudate and putamen; *Tu*, olfactory tubercle; *AV*, anteroventral thalamic nucleus; *Th*, thalamus (laterodorsal and lateral posterior thalamic nuclei, posterior thalamic nuclear, ventroposterior, ventromedial, and ventrolateral thalamic nuclei); *Rt*, reticular thalamic nucleus; *ZI*, zona incerta; *APT*, anterior pretectal area; *FrPaM*, frontoparietal cortex motor area.

Shipley, 1993). In the glomerular layer consisting of periglomerular cells, cRyR mRNA (Fig. 3*B*) was predominantly seen in the superficial part, while bRyR mRNA (Fig. 3*C*) was observed in the deeper half. RyR immunoreactivity was also shown in the glomerular layer of the fish olfactory bulb (Zupanc et al., 1992). cRyR and bRyR mRNAs was found in the external plexiform layer consisting of tufted cells. In the mitral cell and granule cell layers, the mRNAs of all three receptor types were observed. In other parts of the olfactory system, the anterior olfactory nucleus, piriform cortex, and olfactory tubercle expressed all three receptor types, but cRyR expression was predominant.

Cerebral cortex. The labeling density of cRyR mRNA was obviously greater than that of the other RyR mRNAs (Figs. 1*C*; 2*B,E*), and showed a laminar distribution (Fig. 4*B*), especially in the frontoparietal cortex, a very high level in layer VI, a moderate level in layers II–III, and a low level in layers I, IV, and V. Low-level expression of sRyR (Figs. 1*A*; 2*A,D*; 4*A*) and bRyR mRNAs (Figs. 1*C*; 2*C,F*; 4*C*) was observed with no significant laminar dominance.

Hippocampal formation. Although the mRNAs of all three receptor types were expressed differentially in the CA1–CA4 pyramidal cell layers of Ammon's horn, the dentate gyrus, the polymorphic region, the subiculum, and the entorhinal cortex (Figs. 1, 2), cRyR predominance was striking in all of these areas (Figs. 1*B*; 2*B,E*). The highest labeling intensity was seen over the dentate granule cell layer (Figs. 5*E*, 6*A*). sRyR mRNA was present almost uniformly in the CA1–CA4 pyramidal cells and dentate granule cells (Figs. 1*A*; 2*A,C*; 5*A,D*), whereas bRyR mRNA expression was much higher in CA1 pyramidal cells than in the other parts of the hippocampus (Figs. 2*C,F*; 5*C,F*; 6*B*). This CA1 pyramidal cell layer was the predominant site for the labeling of bRyR mRNA (Fig. 1*C*).

Basal ganglia. As shown in Figures 1, 2, and 6, both cRyR and bRyR mRNA were detected in the caudate, putamen, and accumbens nucleus, in which medium and large cells lightly counterstained with cresyl violet were labeled (Fig. 7*A,B*). In contrast, sRyR mRNA expression was not so marked. In the substantia nigra, low labeling levels of cRyR mRNA were observed (Fig. 1*B*).

Thalamus. Very low levels of sRyR mRNA were seen in the thalamus (Figs. 1*A*, 2*D*). Either cRyR or bRyR was highly expressed in particular subdivisions of the thalamus (Figs. 1, 2). Intense labeling of cRyR mRNA was seen mainly in the epithalamus (e.g., lateral habenular nucleus) and the ventral thalamus (e.g., reticular thalamic nucleus and zona incerta) (Figs. 2*E*, 6*A*). In marked contrast to cRyR mRNA, bRyR mRNA was found in the dorsal thalamus (Figs. 2*F*, 6*B*), at fairly high densities in the ventral nuclei (medium and large cells lightly

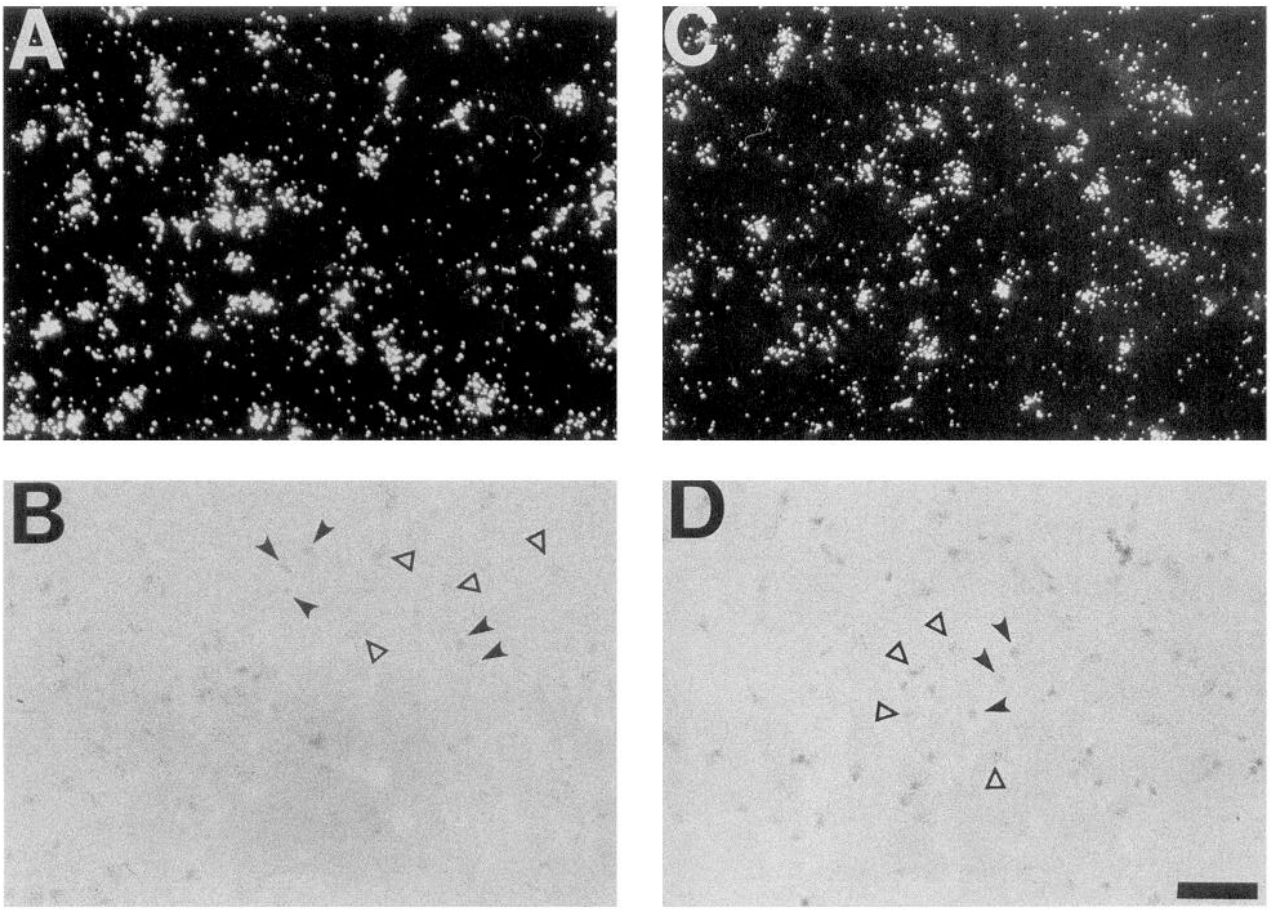

Figure 7. Localization of bRyR mRNA in rabbit caudate, putamen, and thalamus: dark-field (*A* and *C*) and bright-field (*B* and *D*) observations of coronal sections of the caudate and putamen (*A* and *B*) and the thalamus (*C* and *D*) hybridized with ^{35}S-labeled bRyR probe. *Open triangles* and *solid arrowheads* represent positive and negative cells, respectively. Scale bar, 100 μm.

stained with cresyl violet were positive; Fig. 7*C,D*), and at slight densities in the dorsal nuclei.

Cerebellum. In the cerebellar cortex, differences in hybridization patterns with the various probes of the RyR family were very conspicuous (Fig. 8*A–C*), in strong contrast to results with the IP_3R1 probe (Fig. 8*D*). IP_3R1 mRNA was abundantly localized in both the dendrites and somata of Purkinje cells (Furuichi et al., 1989, 1993). sRyR mRNA showed a characteristic high expression level in Purkinje cells (Fig. 9*A,D*), as had previously been demonstrated by immunohistochemical analyses (Ellisman et al., 1990; Walton et al., 1991). A high density of cRyR mRNA was seen in the granule cell layer (Fig. 9*B,E*). cRyR mRNA coexisted with sRyR mRNA in Purkinje cells, and was also present in interneurons of the molecular layer. Labeling for bRyR mRNA was not significant in the cells of the cerebellar cortex (Fig. 9*C,F*), but was slightly above background in the film autoradiogram (Fig. 1*C,D*). Although not in all cells, some bRyR mRNA signals were observed in the granular layer, and a few signals were sometimes seen in Purkinje cells. In the cerebellar nuclei, bRyR mRNA (Fig. 10) coexisted with cRyR mRNA (data not shown).

Other brain regions. As shown in Figures 1 and 2 and Table 1, hybridization signals of the three receptor mRNAs were observed in cells in the hypothalamus, amygdala, superior and inferior colliculus, dorsal cochlear nucleus, external cuneate nucleus, and so on. Most intriguing was the high cRyR expression

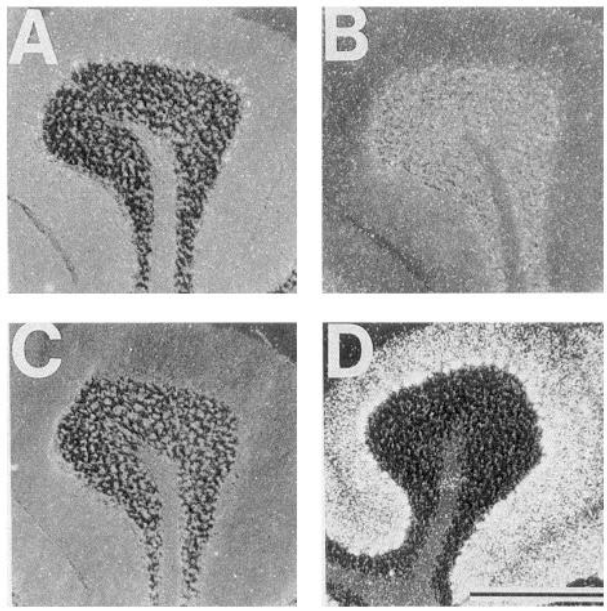

Figure 8. Differential localization of mRNAs of the RyR family and IP_3R1 in rabbit cerebellar lobes: dark-field observations of coronal sections of cerebellar lobes hybridized with ^{35}S-labeled probes of the RyR family (*A*, sRyR; *B*, cRyR; *C*, bRyR) and IP_3R1 (*D*). Scale bar, 500 μm.

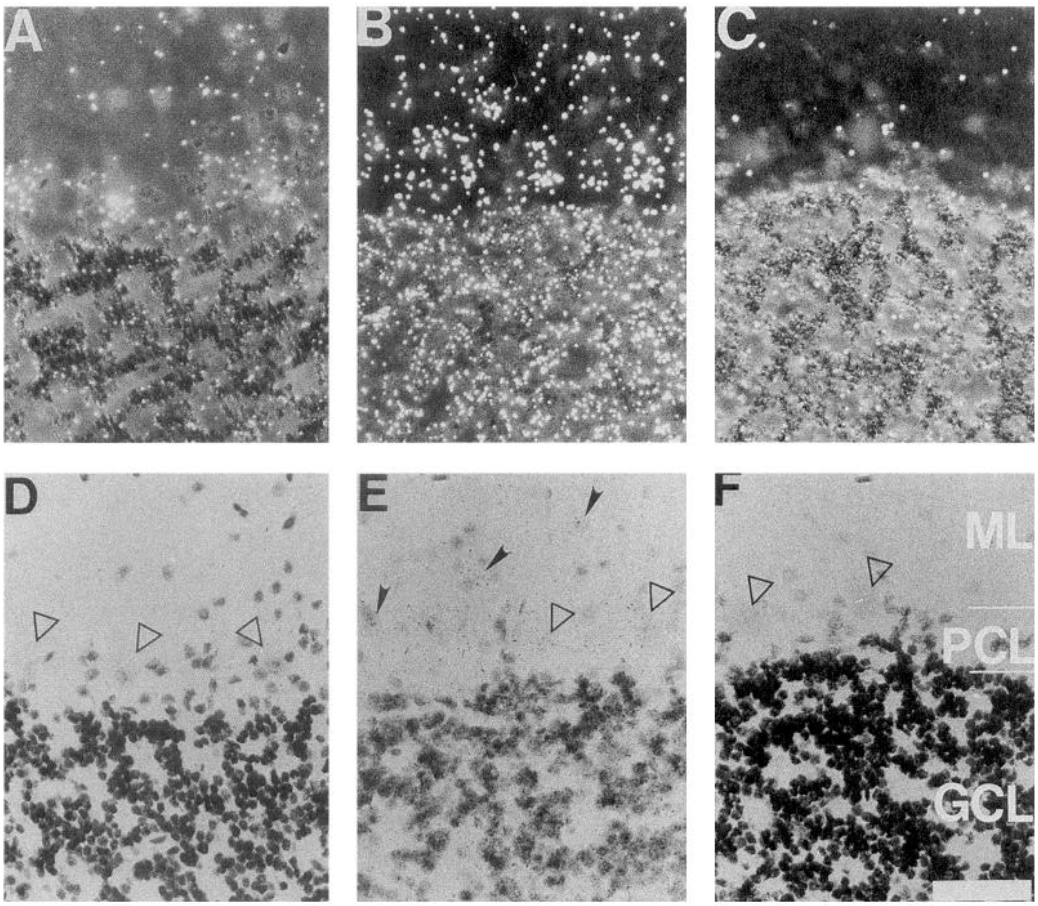

Figure 9. Differential localization of neuronal RyR mRNAs in the rabbit cerebellar cortex (higher magnifications of Fig. 8): dark-field (*A–C*) and bright-field (*D–F*) observations of coronal sections of the cerebellar cortex hybridized with ^{35}S-labeled sRyR (*A* and *D*), cRyR (*B* and *E*), and bRyR (*C* and *F*) probes. *Open triangles* represent Purkinje cell bodies. *Solid arrowheads* point to positive neurons in the molecular layer. *ML*, molecular layer; *PCL*, Purkinje cell layer; *GCL*, granule cell layer. Scale bar, 50 μm.

seen in the motor trigeminal nucleus (Fig. 11*A,D*), facial nucleus (Fig. 11*B,E*), and lateral reticular nucleus (Fig. 11*C,F*). bRyR mRNA also coexisted in these nuclei (for examples, see Fig. 1*C*). Intermediate labeling intensity of cRyR mRNA was observed in the nucleus of the spinal tract trigeminal nerve (Fig. 12). A similar labeling pattern of sRyR and bRyR mRNAs was seen at low-level densities (data not shown).

Discussion

The rabbit neuronal RyR family is composed of three distinct types: bRyR, which was recently isolated from the brain (Hakamata et al., 1992), and sRyR and cRyR, which were originally well characterized as muscle types. These neuronal RyRs are differentially expressed in various brain areas, and are distinguished by their own peculiar distribution in particular brain areas, such as the olfactory bulb, cerebral cortex, hippocampus, thalamus, and cerebellar cortex. This heterogeneous distribution of multiple neuronal RyRs throughout the brain suggests their differential roles in brain function. In addition, these three distinct types of RyRs coexist within individual neurons, such as hippocampal CA1 pyramidal cells. Both sRyR and cRyR are known to function as homotetrameric receptor/channel complexes (Lai et al., 1988; Anderson et al., 1989). bRyR seems to form an analogous tetramer. Therefore, the coexpression of these distinct RyR types in a cell suggests that neuronal RyR complexes may be made up of more than one subunit type arranged in a tetrameric structure. Receptor/channel kinetics of heteromeric RyR complexes could vary depending upon these different subunit combinations, thereby modulating neuronal RyRs involved in a variety of brain functions. On the other hand, these organellar channels could be located in spatially different Ca^{2+} stores involved in differential Ca^{2+} signaling within individual cells (i.e., dendritic, axonal, and/or somatic Ca^{2+} storage organelles), which is inferred from the observation that in cerebellar Purkinje cells, the localization of immunoreactivity of chicken sRyR (Walton et al., 1991) and rat cRyR (Sharp et al., 1993) appears to be spatially different from that of the other class of the organellar channel family, IP_3R1.

Recently, the channel properties of caffeine-sensitive and ryanodine-sensitive Ca^{2+} release channels in the brain have been examined by various systems (Ashley, 1989; Bezprozvanny et al., 1991; McPherson et al., 1991; Lai et al., 1992). These results suggested that neuronal RyR is almost indistinguishable pharmacologically (sensitivity to caffeine, ryanodine, Ca^{2+}, and ATP) from the RyRs in a variety of muscle tissues, but is slightly different in electrophysiological properties (e.g., channel conductance). It is now apparent, however, that in these studies the authors probably recorded channel activities of a complex of neuronal RyR channels and that most of this activity was attributable to cRyR channels, which are dominant throughout

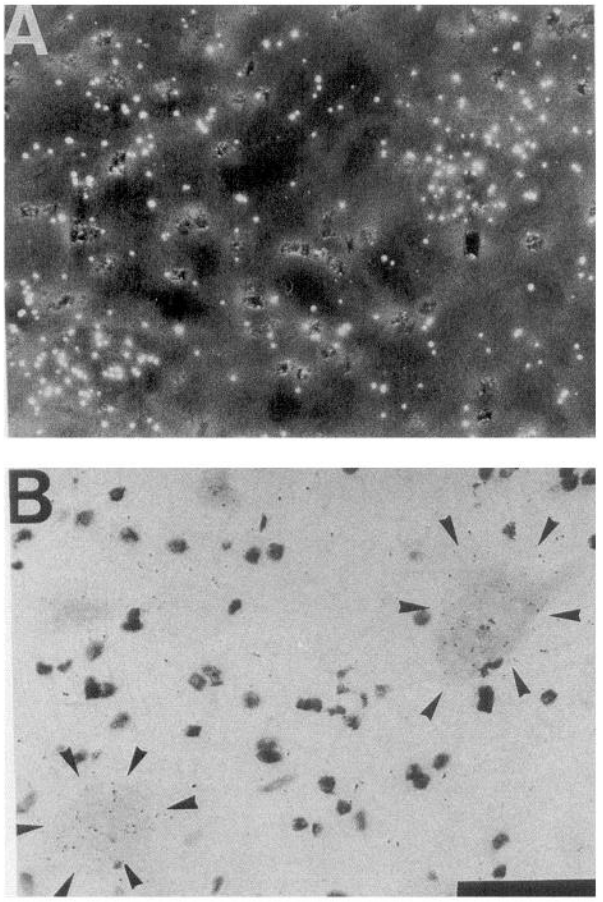

Figure 10. Localization of bRyR mRNA in rabbit cerebellar nuclei: dark-field (*A*) and bright-field (*B*) observations of sagittal sections of cerebellar nuclei hybridized with ^{35}S-labeled bRyR probe. The *arrowheads* in *B* are pointing to large cells with many positive grains. Scale bar, 50 μm.

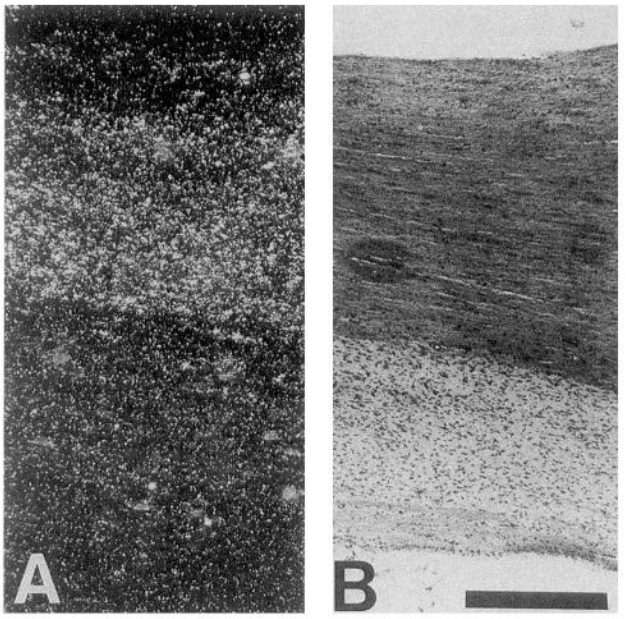

Figure 12. Localization of cRyR mRNA in the spinal tract of the rabbit brain: dark-field (*A*) and bright-field (*B*) observations of sagittal sections of spinal tracts hybridized with ^{35}S-labeled cRyR probe. Scale bar, 500 μm.

most of the brain. cRyR functions as a Ca^{2+}-induced Ca^{2+} release (CICR) channel in cardiac muscle (Fabiato and Fabiato, 1973; Fabiato, 1985). It is believed that in central neurons Ca^{2+} entry through voltage-activated Ca^{2+} channels or Ca^{2+}-permeable ligand-gated ion channels is the primary signal for initiating the $[Ca^{2+}]_i$ transient, and that CICR probably amplifies the effects of Ca^{2+} influx (Bliss and Collingridge, 1993). This regenerative $[Ca^{2+}]_i$ rise by CICR is thought to be involved in

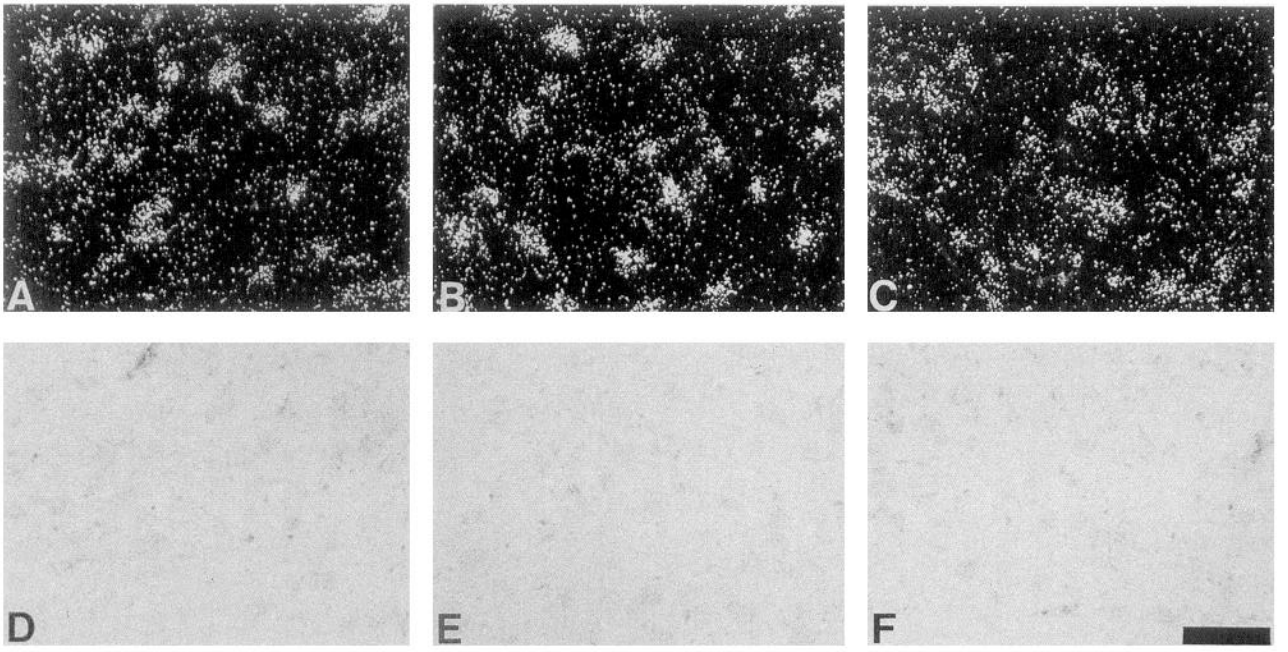

Figure 11. Localization of cRyR mRNA in the facial, motor trigeminal, and lateral reticular nuclei of the rabbit brain: dark-field (*A–C*) and bright-field (*D–F*) observations of sagittal sections hybridized with ^{35}S-labeled cRyR probe. *A* and *D*, facial nucleus; *B* and *E*, motor trigeminal nucleus; *C* and *F*, lateral reticular nucleus. Scale bar, 50 μm.

oscillatory $[Ca^{2+}]_i$ elevation in a cell in some cases. We showed the widespread localization of cRyR in rabbit brain, suggesting that upon the primary $[Ca^{2+}]_i$ elevation, the CICR events could occur throughout the brain. The present results also indicate that sRyR and bRyR are only a fraction of neuronal RyR and have the expression pattern distinct from that of cRyR. Purkinje cells, which highly express sRyR mRNA, are known to possess subsurface cisternae just beneath the plasma membrane, like the sarcoplasmic reticulum of skeletal muscle (Henkart et al., 1976), and some fractions of sRyR proteins in Purkinje cells exist in these subsurface membrane cisternae (Walton et al., 1991). Thus, the neuronal sRyR in Purkinje cells may form a "foot"-like structure, thereby opened through some mechanical interaction with high-voltage-activated Ca^{2+} channels, just like the model in skeletal muscle (Schneider and Chandler, 1973; Catterall, 1991). It has been well documented that the opening of both sRyR and cRyR channels is stimulated by both ryanodine and caffeine. In contrast, bRyR appears to release Ca^{2+} in response to ryanodine but not to caffeine (Giannini et al., 1992). Thus, bRyR appears to have a receptor/channel property distinct from that of sRyR and cRyR. Finally, the results of recent intriguing studies on intracellular Ca^{2+} release have suggested that cyclic adenosine diphosphate ribose (cADPR) is a major candidate(s) for the endogenous ligand of RyRs (Galione, 1992, 1993; Lee, 1993; Takasawa et al., 1993; White et al., 1993). Indeed, extracts from rabbit brain contain cADPR-synthesizing enzymes (Rusinko and Lee, 1989). Therefore, some of neuronal RyR-mediated $[Ca^{2+}]_i$ increases may be triggered by this putative intracellular messenger, cADPR.

In conclusion, the present data indicate that each functionally divergent type of neuronal RyR family is differentially localized in the rabbit brain and appears to be involved in the amplification of Ca^{2+} signals by CICR or may transduce electrical information (membrane depolarization) or intracellular messenger(s), such as cADPR, into Ca^{2+} signals.

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