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Differential distribution of the alternative forms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, SERCA2b and SERCA2a, in the avian brain

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Cellular distribution of the two forms of SERCA2 was examined in adult chicken brain. Four regions of the brain were analyzed with three immunological reagents: a monoclonal antibody that recognizes both forms of SERCA2, and two antisera which are specific for the two alternative forms, SERCA2b or SERCA2a. Cerebellar Purkinje cells express predominately SERCA2b but also low levels of SERCA2a, as has been reported for mammals[22]. The nucleus isthmo-opticus, nucleus magnocellularis cochlearis, and nucleus laminaris all express high levels of SERCA2 but with different ratios of SERCA2b and SERCA2a. These immunohistochemical results were supported by in situ hybridization analysis. Therefore, it appears that regions within the brain have specific requirements for the two forms of SERCA2. This suggests functional significance for the alternative forms SERCA2b and SERCA2a, and possible functions are discussed.

INTRODUCTION

The cytosolic concentration of free Ca^{2+} is critical for many aspects of neuronal functions[3,17]. Neurons, like many cells, often maintain more than one intracellular pool of Ca^{2+}[2] and presumably use each of these pools in specific regulatory events. In order for a membrane bound compartment to function as a Ca^{2+} pool, it must contain three components: a Ca^{2+} release channel, a Ca^{2+} buffer, and a Ca^{2+}-ATPase to replenish the depleted pool.

Some neurons (e.g. cerebellar Purkinje cells (PCs)) have at least two intracellular pools of Ca^{2+}, though the function(s) of the two pools is unknown. Several elegant studies[25,27,28] have demonstrated that chicken PCs express two distinct Ca^{2+} release channels, IP3 receptors and ryanodine receptors. These Ca^{2+} pools have been shown to contain calsequestrin, a calcium binding protein which acts as a Ca^{2+} buffer, and the slow-twitch or cardiac form of the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+-} ATPase (hereafter referred to as SERCA2). Plessers et al.[22] reported that porcine PCs expressed predominantly the longer alternative form of SERCA2 (SERCA2b) in addition to low levels of the shorter alternative SERCA2 form (SERCA2a). These two forms of SERCA2 differ at their extreme carboxyl termini. The 49 terminal amino acids of SERCA2b are replaced by 4 amino acids in SERCA2a. These alternative termini are due to alternative splicing of a common primary transcript derived form the same gene[6,8]. These recent investigations have highlighted PCs as model neurons to use in studies concerning Ca^{2+} regulation.

In order to gain a broader understanding of Ca^{2+} regulation throughout the brain, we have undertaken a qualitative study of the chicken brain to answer several questions: (1) do avian PCs express more SERCA2b than SERCA2a, as reported for mammals? (2) Are there other neurons within the brain which also express high levels of SERCA2? (3) If so, do these

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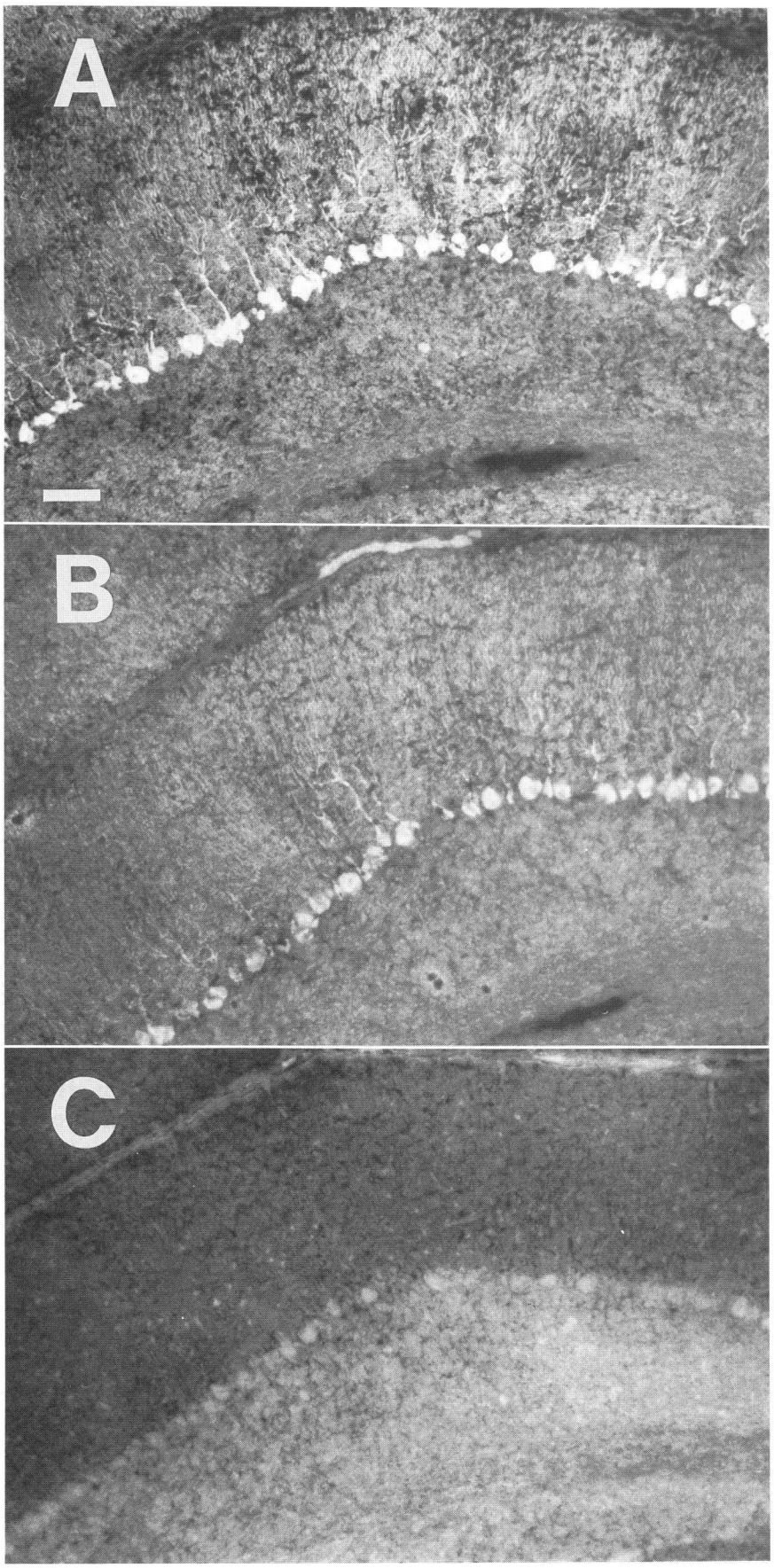
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neurons express more SERCA2b than SERCA2a like PCs do, or does each cell type have a unique SERCA2b/SERCA2a ratio?

MATERIALS AND METHODS

Antibodies

The avian SERCA2 specific but subtype indiscriminate monoclonal antibody (mAb) 3H2 has been previously described[14]. The antisera specific to the carboxyl terminus of SERCA2a[9] and the carboxyl terminus of SERCA2b[29] were prepared as described. Because avian and porcine SERCA2b and SERCA2a have identical terminal amino acid sequences[6,8] we were able to use the same SERCA2b-specific and SERCA2a-specific antisera.

Immunofluorescence microscopy

After the animals had been anesthetized, fresh chicken brains were obtained from chickens belonging to Dr. Saul Roseman. The brains were dissected and then embedded in OCT (Miles Inc., Elkhart, IN) and frozen quickly on dry ice. Ten-μm-thick sagittal sections were made on a Slee (London) cryostat and mounted on subbed slides (0.05% chromic potassium sulfate dodecahydrate, 0.3% gelatin, 0.04% sodium azide). The tissue was surrounded with rubber cement to reduce reagent volume and treated at room temperature as follows: fixed in buffered (25 mM HEPES pH 7.4, 2.5 mM Mg acetate, 25 mM KCl, 250 mM sucrose) 4% formaldehyde for 1 h, blocked with buffered 0.25% gelatin and 5μg/ml lysine for 30 min, incubated for 1 h with the primary antibody (5μg/ml of 3 H_2 or 1:100 dilution of polyclonal antiserum) in buffered 0.25% gelatin and 0.2% Triton X-100 (Sigma) for 1 h, washed three times 2 min with buffer, incubated for 1 h with buffered FITC conjugated secondary antibody, washed with buffer for 3 times 10 min, and covered with 30 μl mounting medium (90% glycerol, 10% PBS and 0.1% paraphenylene diamine) and a coverslip. Immunofluorescence photomicrographs were taken with a Zeiss Axioskop. For comparative purposes, sections from the same specimen blocks were cut and processed in parallel for fluorescence microscopy. Negative controls for the immunofluorescence consisted of sections processed in parallel but with omission of the primary antibody and substitution of an irrelevant monoclonal antibody for mAb 3 H_2. Photographs were taken under identical conditions of illumination. Negatives were printed with identical parameters so that differences appearing between panels in each figure reflect differences in the appearance of the specimens.

In situ hybridization

Fresh tissue was collected as described above on the day before experiments were performed in order to maximize mRNA levels. For the cRNA probes, 10-μm-thick sagittal cryosections were used while 16-μm-thick sagittal sections were used for the end-labeled oligonucleotide probes. All sections were prepared, prehybridized and hybridized as described[24]. The cRNA probes were hybridized and washed at 52°C while oligonucleotide probes were hybridized and washed at 45°C.

cRNA probe production

PCR amplified cDNA of chicken SERCA2 (between bases 1120 to 1270; Campbell) were cloned into pBluescript II SK-(Stratagene) via the Sac I and Kpn I sites incorporated into the 5′ and 3′ primers, respectively. This 150 base pair fragment encoded a central portion of SERCA2 that was common to both SERCA2b and SERCA2a, but was very dissimilar to chicken SERCA1[15] and rat SERCA3[4]. Boehringer Mannheim's RNA labeling kit was used as recommended by the manufacturer to generate the antisense probe (T7 RNA polymerase) and the sense strand, negative control, probe (T3 RNA polymerase). This method incorporated about three digoxigenin-11-UTPs per probe, which was detected with Boehringer Mannheim's anti-digoxigenin alkaline phosphatase conjugated antibody. The color reaction was performed in the presence of levamisole (Sigma), 5-bromo-4-chloro-3-indolyl-phosphate and Nitro blue tetrazolium (Boehringer Mannheim) as described[24].

End-labeled oligonucleotide probe production

Due to the alternative splicing mechanism utilized to produce SERCA2b and SERCA2a mRNA, all of SERCA2a encoding mRNA is also contained within SERCA2b mRNA. Therefore, the only way to produce SERCA2b- and SERCA2a-specific probes for in situ hybridization was to design oligonucleotides which span the alternative splice site[6]. These two oligonucleotides were 60 bases long and comprised of two halves: the 5′ halves were complementary to 30 bases of mRNA encoding the carboxyl termini of SERCA2b or SERCA2a while the 3′ halves were identical to each other but complementary to a common domain of SERCA2, upstream of the alternative splice site. The sequences were: SERCA2b: 5′ GTTTGGTGGCAGGCTGCACACTATCTTTACCAGGTTCCAGGTAGTTACGGGCCACGTATT 3′, and SERCA2a: 5′ ATTGTTTAGGAAGTGATTACTCCAGTATTGCAGGTTCCAGGTAGTTACGGGCCACGTATT 3′ Under the hybridization and wash conditions used, the calculated T_m^7 of the oligonucleotide probes was 59°C for SERCA2b, and 56°C for SERCA2a. One hundred pmol of oligonucleotide was 3′ end-labeled using terminal nucleotide transferase so that a single digoxigenin-11-dideoxy-UT was added onto each oligonucleotide, as recommended by Boehringer Mannheim. Specific binding was assessed by comparing labeled serial sections prepared with or without 20-fold excess of unlabeled oligonucleotide probe added to the hybridization mixture.

RESULTS

When the cerebellum was labeled with the three immunological reagents (Fig. 1), a striking pattern of labeling was revealed. mAb 3 H_2 labeled the PCs very intensely with only moderate levels of labeling apparent in the granular cell layer, as previously reported[13]. The SERCA2b-specific antiserum clearly labeled the PCs but there was no detectable labeling in the granular layer. In contrast, the SERCA2a-specific antiserum labeled the PCs weakly while there was bright labeling of the granular layer. The PCs' SERCA2b/SERCA2a ratio of labeling was consistent with Plessers et al.[22]. Others have also reported SERCA2 expression in granular layer cells[25,26]. However, the SERCA2a-specific labeling of the granular cell layer has not been reported previously. If the anti-SERCA2a labeling of the granular cells were non-specific, then this non-specific labeling was confined to the granular cell layer of the cerebellum alone. Since it is known that SERCA2 is expressed in these cells and SERCA2b-specific labeling was undetectable, it must be assumed that these cells do indeed express predominantly SERCA2a.

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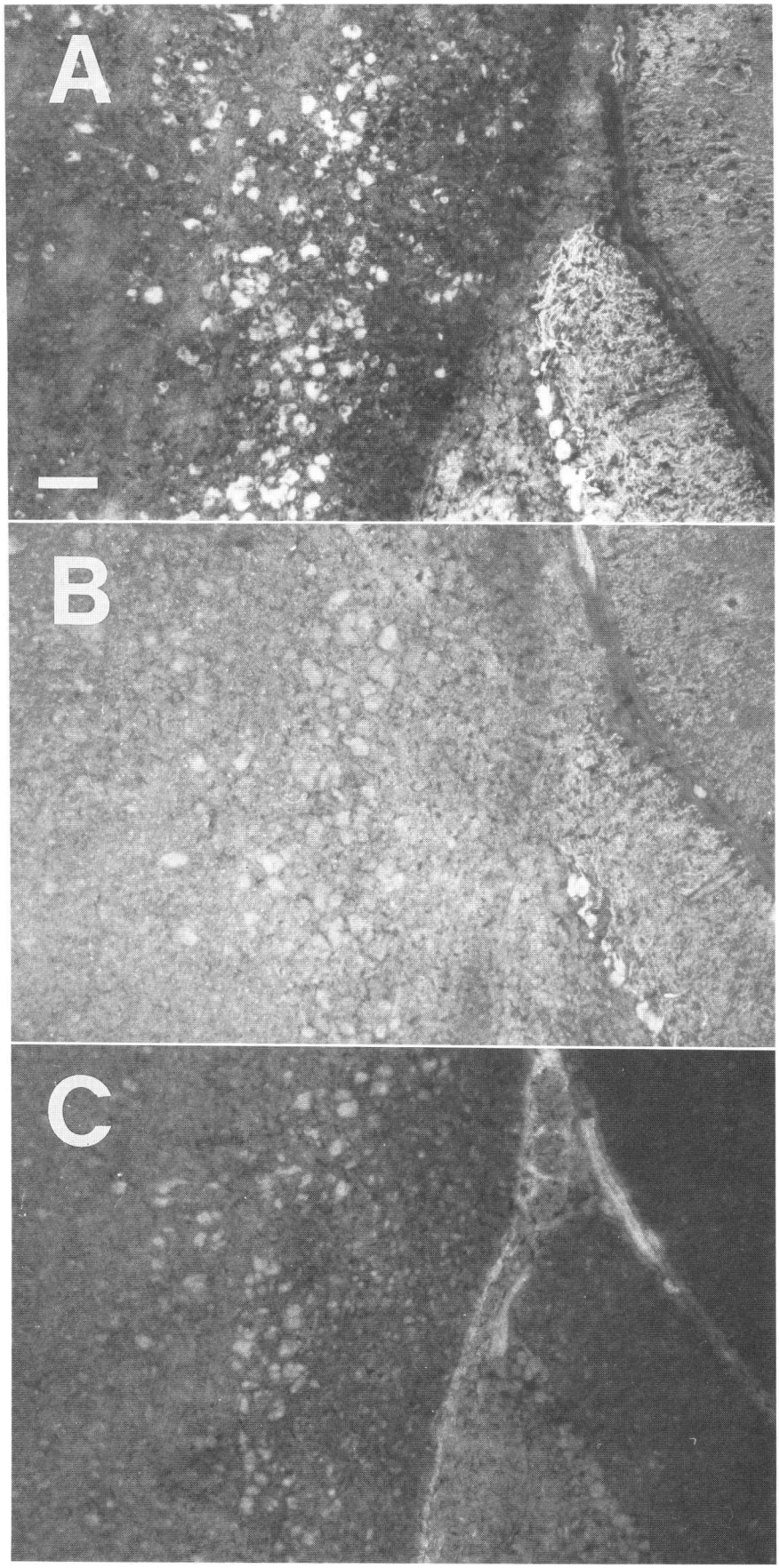
Fig. 1. Immunofluorescence micrographs of adult chicken cerebellum labeled with (A) anti-SERCA2 subtype-indiscriminate mAb 3 H_2, (B) SERCA2b-specific or (C) SERCA2a-specific antisera. Note the faint labeling of cell bodies and dendritic processes in C. Bar in A = 100 μm and applies to all three panels.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The anti-avian SERCA2 mAb 3 H_2 was used on cryosections to find non-cerebellar regions within the brain which expressed high levels of SERCA2. There were many neurons which expressed high levels of SERCA2 but we decided to limit this investigation to the cerebellum and three nearby nuclei which could be contained within a single section and easily identified. The nuclei examined in detail were the nucleus isthmo-opticus (IO), nucleus magnocellularis cochlearis (MCC), and nucleus laminaris (La)[17]. These three nuclei and the cerebellum were examined subsequently with the SERCA2b- and SERCA2a-specific antisera.

A series of immunofluorescence micrographs of the IO is shown in Fig. 2. As is evident (Fig. 2A), neurons within this nucleus express high levels of SERCA2. In contrast to the cerebellum (see Figs. 1 and 2), it appears that there might be slightly more SERCA2a expressed in these neurons than SERCA2b. The neurons of neighboring nuclei MCC and La (Figs. 3 and 4) also express high levels of SERCA2 but with opposite SERCA2b/SERCA2a ratios. It appears that MCC might express more SERCA2a than it does SERCA2b. Conversely, La appears to express more SERCA2b than SERCA2a.

To corroborate these data, in situ hybridization analysis was performed. Three digoxygenin labeled probes were used (see Materials and Methods) which were analogous to the immunological probes in that one was SERCA2b-specific, one SERCA2a-specific and the third labeled both forms of SERCA2. In the cerebellum (Fig. 5), there was a high degree of labeling of mRNA within PCs with the SERCA2-indiscriminate, SERCA2b-specific, and SERCA2a-specific probes. This supported the immunohistochemical data that PCs express both SERCA2b and SERCA2a. However, there was no significant labeling of mRNA in the granular cell layer with any of the probes. Since granular layer cells do express SERCA2 protein, they must at some time also transcribe SERCA2 encoding mRNA. The lack of detectable labeling might be due to a low level of SERCA2 mRNA in adult granular cells. This is consistent with previous work[1,12] which has shown both temporal and spacial variations in accumulation of a calcium binding protein and its encoding mRNA within the brain.

The IO neurons (Fig. 6) labeled well with all three probes, confirming the antibody data that demonstrated a mixture of SERCA2b and SERCA2a within these neurons. Likewise, the MCC neurons also labeled with all three probes. In the La however, there appeared to be weakly positive labeling with the SERCA2-indiscriminate and SERCA2b-specific probes, but the SERCA2a-specific probe resulted in background levels of labeling, compared to control levels (data not shown, compare with Fig. 5). These data are consistent with the immunological data presented above.

DISCUSSION

Neurons of the cerebellum, IO, MCC and La expressed high levels of SERCA2 and each region studied appeared to express unique ratios of SERCA2b and SERCA2a. Since it was first reported that SERCA2 was expressed as two forms[11,19], investigators have tried unsuccessfully to identify functional differences between SERCA2b and SERCA2a[6,20]. To date, the only reported difference between the two forms is that SERCA2b and SERCA2a have their carboxyl termini on opposite sides of the SR/ER membrane[5]. From the data presented here, it is clear that PCs are not the only neurons to co-express SERCA2b and SERCA2a. Do such neurons have a way to regulate differentially the function of the two forms of SERCA2? If so, it is most probably done through a mechanism that involves the alternative carboxyl termini.

Why have the carboxyl termini of SERCA2b and SERCA2a been conserved in birds and mammals through the millions of years since their evolutionary divergence? There must have been some selective (functional) pressure to maintain SERCA2b and SERCA2a. Perhaps their functional roles are related to the different intracellular pools of Ca^{2+}. Interestingly, SERCA2a expression correlates with the ryanodine receptor while SERCA2b correlates with the IP3 receptor. For example, heart preferentially expresses SERCA2a and ryanodine receptor[6,9,10,29]; PCs express predominantly SERCA2b and IP3 receptor but also low levels of SERCA2a and ryanodine receptor[6,22,23,29]. Walton et al.[29] have co-localized these different Ca^{2+} pools via their receptors using immunological methods at the electron microscope level. Based on these results, we hypothesize that the relative amounts of IP3 receptor and ryanodine receptor would correlated with the relative amounts of SERCA2b and SERCA2a expressed in the IO, MCC and La. Furthermore, we

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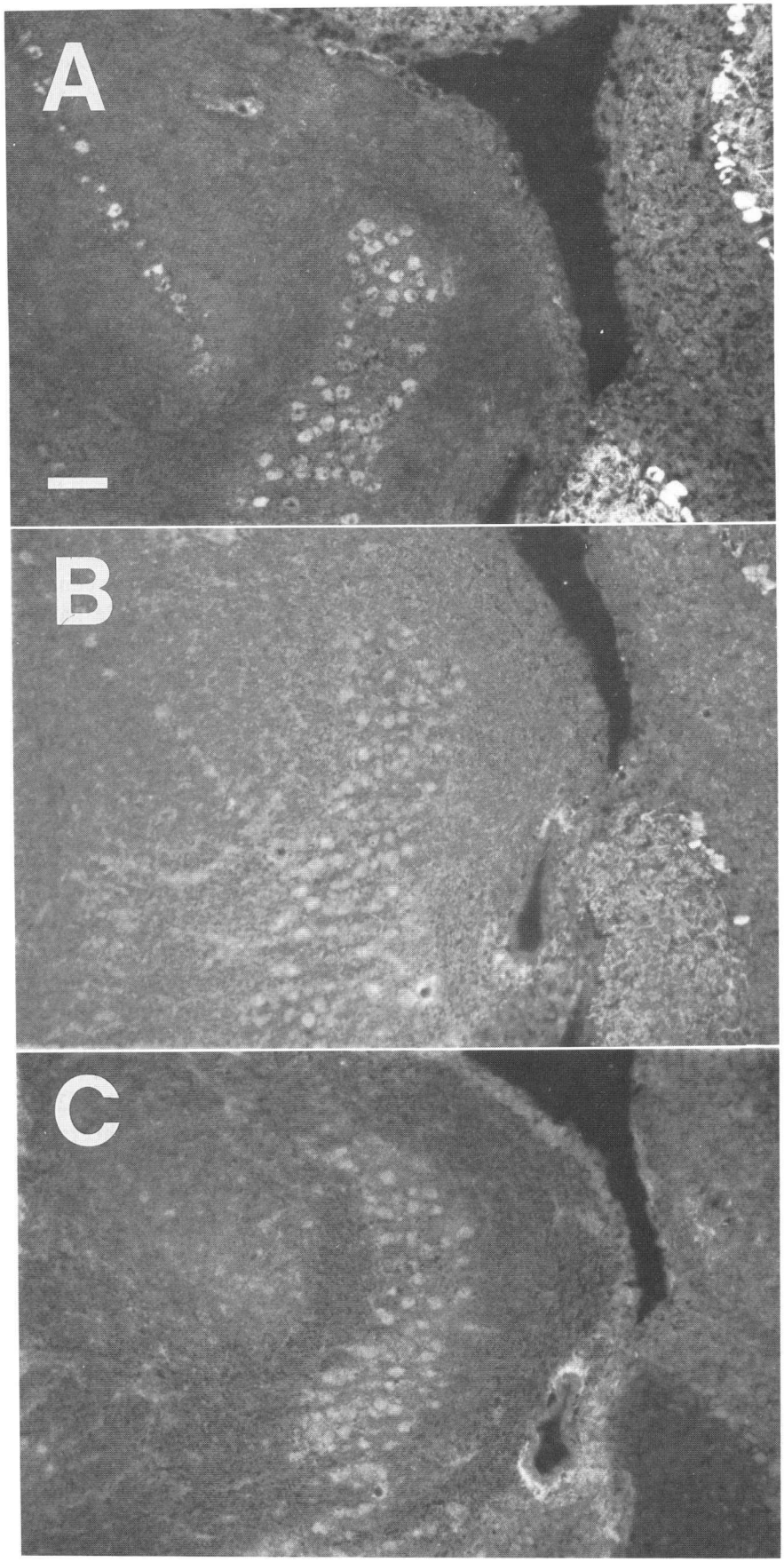
Fig. 2. Immunofluorescence micrographs of nucleus isthmo-opticus (IO) labeled with (A) anti-SERCA2 subtype-indiscriminate mAb 3 H_2, (B) SERCA2b-specific or (C) SERCA2a-specific antisera. Note the cerebellar Purkinje cells in the lower right hand corner of all three panels. Bar in A = 100 μm and applies to all three panels.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predict that an EM immunohystochemical study of PCs, IO, MCC and La would reveal that SERCA2b co-localized with the IP3 receptor and SERCA2a co-localized with the ryanodine receptor. Perhaps the two alternative carboxyl termini of SERCA2 are involved in partitioning the different Ca^{2+}-ATPases to segregate with the appropriate Ca^{2+} release channel. Although this hypothesis is speculative, it is based on correlation with a similar E1-E2 ATPase: the Na/K-ATPase. The α subunit of the Na/K-ATPase uses its carboxyl terminus to interact with the β subunit and this carboxyl terminal interaction does not interfere with enzyme activity[18].

Before potential interactions between SERCA2 and Ca^{2+} release channels could be studied, immunological studies of IP3 and ryanodine receptors' distribution in the brain should be conducted to see if high levels of IP3 and ryanodine receptors are express in IO, MCC and La. Then, subcellular localization of SERCA2b and SERCA2a with respect to IP3 and ryanodine receptors could be determined for neurons of these nuclei. By examining such issues we should better understand the origin, maintenance and function of intracellular Ca^{2+} pools throughout the brain.

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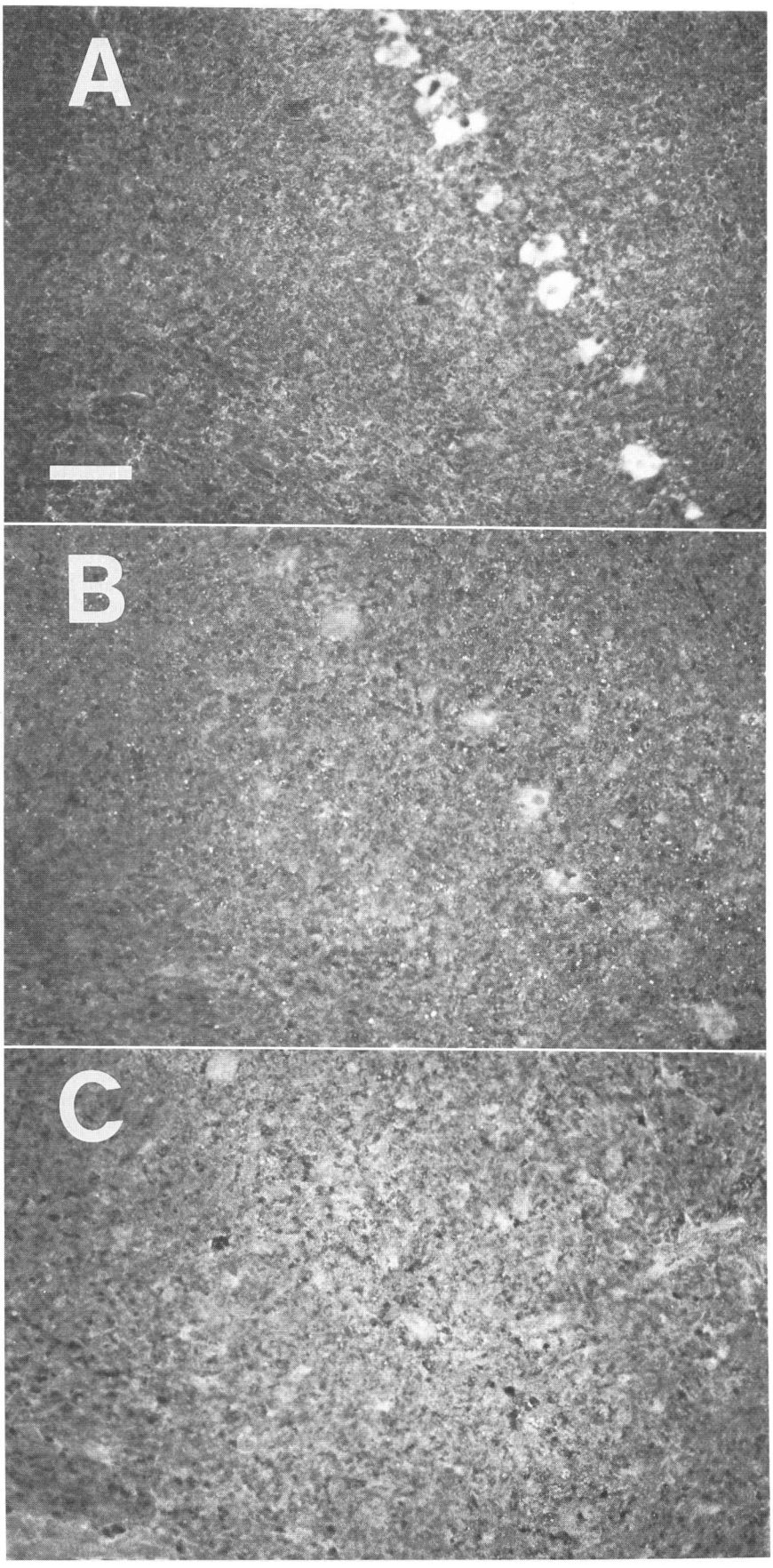
Fig. 3. Immunofluorescence micrographs of nucleus magnocellularis cochlearis (MCC), and nucleus laminaris (La) labeled with (A) anti-SERCA2 subtype-indiscriminate mAb 3 H_2, (B) SERCA2b-specific or (C) SERCA2a-specific antisera. The MCC is the crescent shaped cluster of cells and the La is the line of cells emerging from the top left corner of each panel. Note the cerebellar Purkinje cells on the lower right side of all three panels. Bar in A = 100 μm and applies to all three panels.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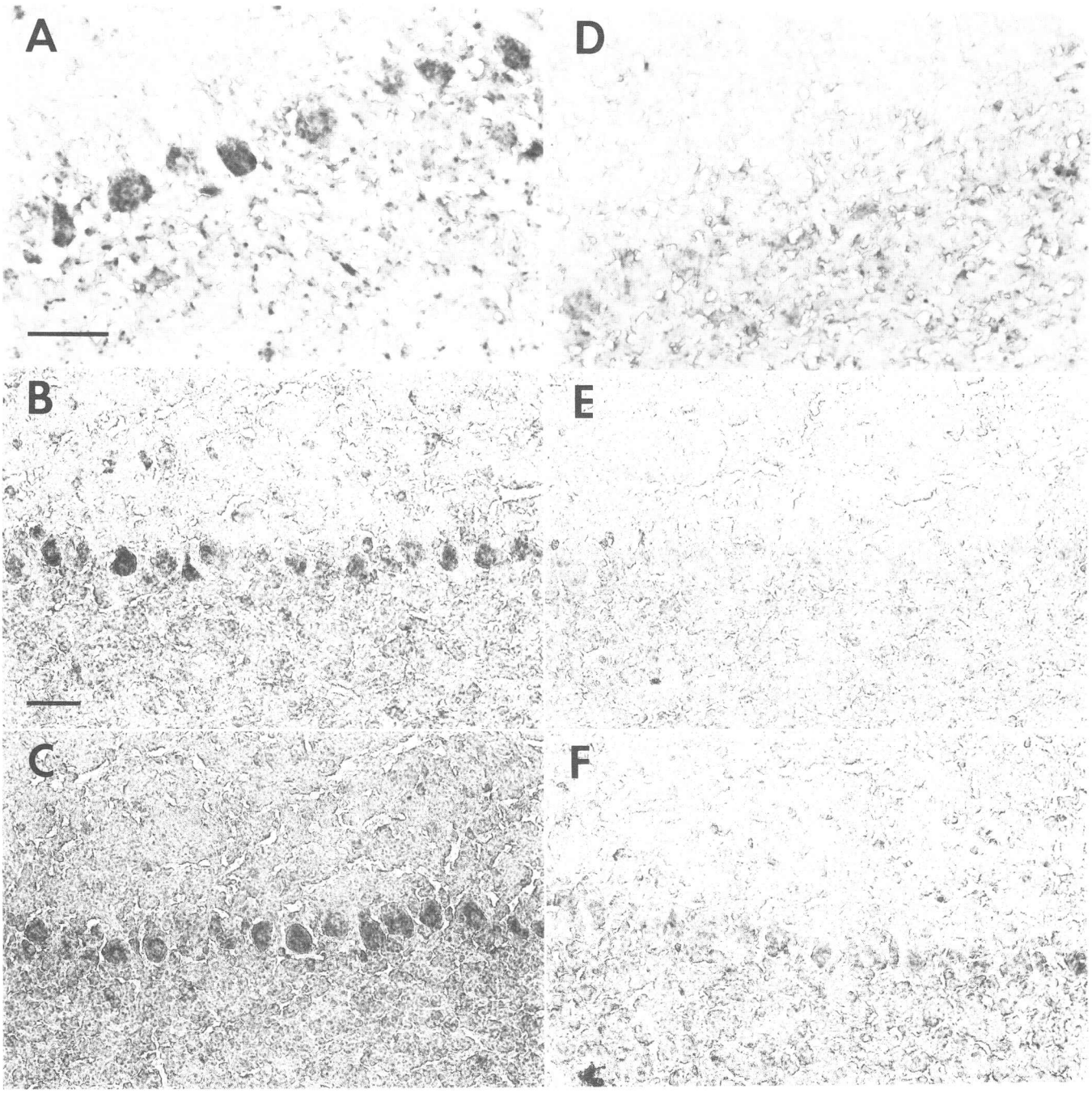

Fig. 5. Photomicrographs of in situ hybridization analysis of chicken cerebellum using the following probes: (A) SERCA2 subtype-indiscriminate (B) SERCA2b- or (C) SERCA2a-specific mRNA. Panels D, E, and F are the controls (see Materials and Methods) for panels A, B, and C, respectively. Bar in A = 50 μm and applies to panels A and D; bar in B = 50 μm and applies to panels B, C, E, and F.

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Fig. 4. Immunofluorescence micrographs of nucleus laminaris (La) at higher magnification than in Fig. 3. Each panel has been labeled with (A) anti-SERCA2 subtype-indiscriminate mAb 3 H_2, (B) SERCA2b-specific or (C) SERCA2a-specific antisera. Bar in A = 50 μm and applies to all three panels.

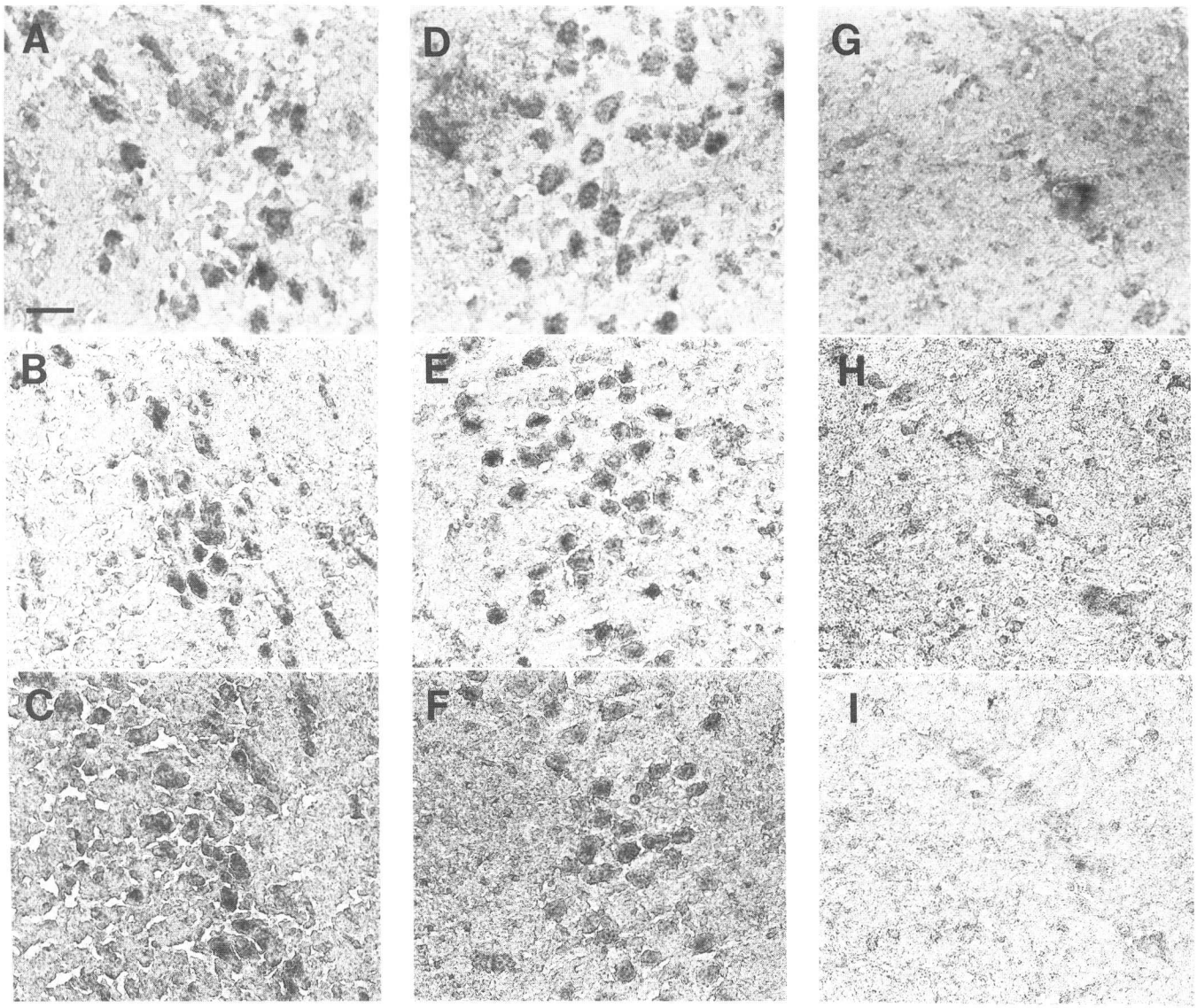

Fig. 6. Photomicrographs of in situ hybridization analysis of chicken nuclei of hte brain using the following probes: (A, D and G) SERCA2 subtype-indiscriminate cRNA; (B, E, and H) SERCA2b-specific oligonucleotide; and (C, F, and I) SERCA2a-specific oligonucleotide. The nuclei in each panel are: A–C, isthmo-opticus (IO); D–F magnocellularis cochlearis (MCC); and G–I, laminaris (La). Bar in A = 50 μm and applies to all panels. Negative controls are not shown but were similar to those in Fig. 5 (see Materials and Methods).

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