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Expression of SV2 in the Developing Chick Cerebellum: Comparison with Calbindin and AMPA Glutamate Receptors 2/3

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The well-organized cerebellum is an ideal model to investigate the developmental appearance and localization of pre- and postsynaptic structures. One of the synaptic proteins abundant in the central nervous system and localized in presynaptic vesicle membranes is the synaptic vesicle protein 2 (SV2). SV2 was shown to be involved in priming and modulating synaptic vesicles and having an effect in epileptic diseases. So far there are no data available describing the developmental localization of this protein in the cerebellum. We followed the expression pattern of SV2 and compared it with the expression of the neuronal calcium-binding protein Calbindin and the AMPA glutamate receptor subunits 2/3 (GluR 2/3), both shown to be early expressed in the developing chick cerebellum predominantly in Purkinje cells. We detected the expression of SV2 in presynaptic terminals (mainly from climbing and mossy fibers) as soon as they are formed at embryonic day 16 in the inner molecular layer. Purkinje cells express Calbindin and GluR 2/3 in the soma and postsynaptically in the primary dendrites at this stage. With ongoing development, the pattern of SV2 expression follows the development of Purkinje cell dendrites in the molecular layer, suggesting a synaptic refinement of labeled climbing and later parallel fibers.

Key words: neuronal development; cerebellum; Purkinje cell; synaptic vesicle; SV2; GluR 2/3

Classic neurotransmitters are stored and released from small synaptic vesicles in the nerve terminals that are endowed with a certain set of membrane proteins necessary for transport and docking of these vesicles to the plasma membrane. One of these ubiquitous and abundant proteins present on all synaptic and secretory vesicles is the synaptic vesicle protein 2 (SV2; Buckley and Kelly, 1985). Three SV2 isoforms (SV2A, SV2B, and SV2C) encoded by distinct genes have been identified in rodents, each with a unique distribution in brain (Bajjalieh et al., 1994; Janz and Südhof, 1999). They are important regulators of Ca^{2+}-stimulated synaptic vesicle exocytosis (Janz et al., 1999; Xu and Bajjalieh, 2001). Recently, SV2 was implicated in priming synaptic vesicles in quiescent neurons to act as a positive

Abbreviations used: E = embryonic day; P = posthatching day; SV2 = synaptic vesicle protein 2; Calb = Calbindin; GluR 2/3 = AMPA glutamate receptor subunit 2 + 3; VGlut2 = vesicular glutamate transporter 2; EGL = external granular layer; ML = molecular layer; GL = granular layer; WM = white matter of the cerebellum.

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modulator of synaptic transmission and enhancer of the release probability of synapses (Custer et al., 2006). Additionally, it was shown to be the brain binding site of levetiracetam (LEV), a new antiepileptic drug (Lynch et al., 2004).

SV2 was shown to be abundant in the central nervous system from early embryonic stages in mouse (Marazzi and Buckley, 1993) and in chick (Bergmann et al., 1999; Grabs et al., 2000). However, no data are available that describe the developmental expression of SV2 in the chick central nervous system, including the cerebellar cortex, a brain region involved in epileptic disorders (Messerschmidt et al., 2008). We used the calcium-binding protein Calbindin as a marker for Purkinje cells (Celio, 1990), to better follow the development of Purkinje cell processes and localize the SV2 staining. Additionally, it has been shown that developing Purkinje cells differ in the content of postsynaptic glutamatergic receptors from other brain areas (Clark, 2005). In the mouse and rat, Purkinje cells already express AMPA receptors as soon as they are formed (Petralia and Wenthold, 1992; Bergmann et al., 1996; Petralia et al., 1998; Lachamp et al., 2005). Recently, the expression of AMPA receptors in the developing chick cerebellum was demonstrated to start as early as embryonic day (E) 10 and was localized to Purkinje cells (Pires et al., 2006).

The appearance of synaptic vesicles and neurotransmitter receptors as well as their proper integration in the postsynaptic membrane of newly formed synapses has major effects on the morphological and functional development of neurons. To find out about the origin of SV2-positive presynaptic terminals around the Purkinje cell bodies and the dendritic tree, we used double labeling of SV2 and the vesicular glutamate transporter VGlut2. VGlut2 has been shown to label exclusively climbing fibers in the cerebellum (Ichikawa et al., 2002; Hioki et al., 2003). The chick cerebellum provides an attractive model to address this issue, because the cytoarchitecture, synaptic connections, and cellular development of the cerebellar cortex is well structured and documented (Mugnaini and Forstronen, 1967; Foelix and Oppenheim, 1974; Bertossi et al., 1986). Thus, we investigated the expression and localization of presynaptic marker SV2 and its correlation with the neuronal calcium-binding protein Calbindin and the postsynaptic expression pattern of AMPA glutamate receptor subunits 2/3 (GluR 2/3) using double immunolabeling to obtain a better understanding of the developmental appearance of presynaptic structures during cerebellar maturation in the chick.

MATERIALS AND METHODS

Animals

The experiments were carried out in accordance with the guidelines published by the Swiss Academy of Medical Sciences (SAMW) and the Swiss Academy of Natural Sciences (SANW) regarding the use of animals for experimental procedures.

White Leghorn chicks posthatching day (P) 20 and chick embryos (E16–E20, stages 39–45, according to Hamburger and Hamilton, 1951) were used in this study. Embryos and posthatched animals were anesthetized with Nembutal (50 mg/kg body weight) and perfused transcardially with a physiological saline solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) fixative.

Immunohistochemistry

For the preparation of tissue sections, the brains were dissected out and immersion postfixed for 4–6 hr with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissue was mounted with TissueTek embedding medium (Miles, Elkhardt, IN), and rapidly frozen on a mounting block cooled over liquid nitrogen. Frontal cryosections were cut at 10 μm. For immunofluorescence, mouse monoclonal antibody against SV2 (DSHB, Iowa; see Buckley and Kelly, 1985) and rabbit polyclonal antibodies directed against Calbindin (Swant, Switzerland; see Chen et al., 2006), VGlut2 (SYSY, Germany; Wojcik et al., 2004), or AMPA glutamate receptor subunits 2/3 (GluR 2/3; Chemicon/VWR, Switzerland; see Bergmann et al., 1996) were diluted 1:1,000 resp. 1:200 in goat serum dilution buffer (pH 7.4; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 17% normal goat serum, 137 mM NaCl, 2.7 mM KCl, 0.3% Triton X-100). Antigen/antibody complexes were visualized using Alexa488 conjugated goat anti-rabbit and Alexa546 conjugated goat anti-mouse IgGs (for further details, see Bergmann et al., 2000, 2002; Grabs et al., 2000; Bergmann et al., 2002; Grabs and Bergmann, 2005). The sections were examined using a Leitz DM-IRBE microscope equipped with epifluorescence and for each slide, digital pictures were taken for both stainings with a cooled CCD camera (Hamamatsu C4742-95, Hamamatsu Photonics, Japan). Adobe Photoshop CS2 was used to adjust brightness and contrast, as well as to colorize fluorescence.

RESULTS

Expression of SV2 and Calbindin During Embryonic and Posthatching Development of the Cerebellum

Immunolabeling for both, SV2 and Calbindin was followed from E16 (Fig. 1A–C). SV2 labeling was observed close to Purkinje cell bodies and around the developing dendritic tree in the inner part of the developing molecular layer, as well as in the granular layer (Fig. 1A,C). At this stage, Calbindin was present in the Purkinje cell perikarya and in primary dendrites (Fig. 1B,C).

At E19, the Purkinje cells are well organized in one layer between the granular and the molecular layer (Fig. 1D–F). SV2 immunoreactivity was found in the molecular layer and also in the granular layer (Fig. 1D,F). Calbindin depicts the Purkinje cell bodies and their dendritic arbors. Additionally, Calbindin is present in the thin Purkinje cell axon in the granular layer and even better in axons running in the white matter of the cerebellum (Fig. 1E,F).

At P20, SV2 is found throughout the cerebellum. The molecular layer that extends now to the cerebellar surface is heavily immunostained for SV2. Additionally, SV2 staining in the granular layer concentrates in clusters (Fig. 1G,I). The immunoreactivity for Calbindin depicts the cell bodies and the full dendritic tree of Purkinje cells (Fig. 1H,I).

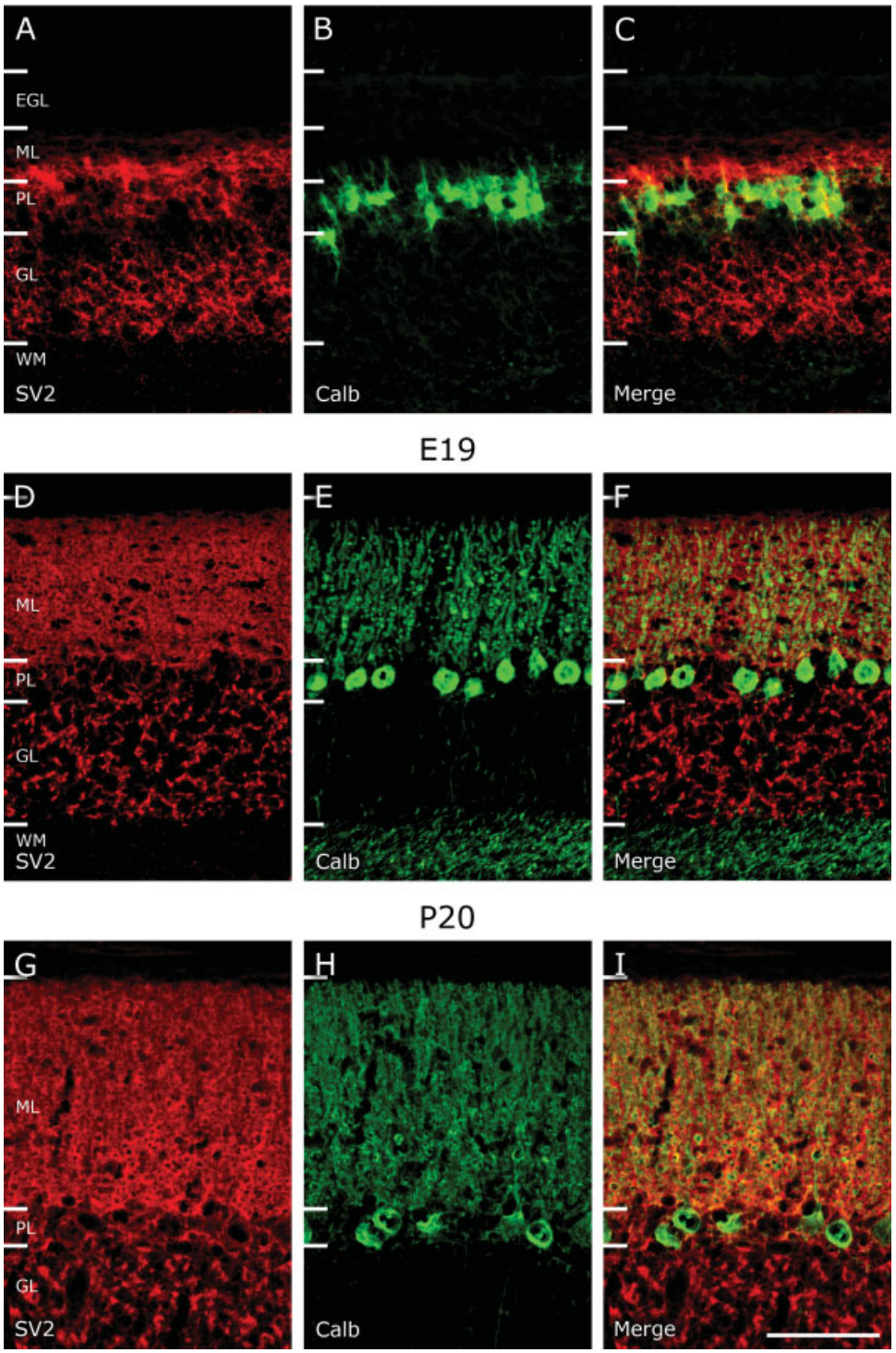

Fig. 1. Expression of synaptic vesicle protein 2 (SV2) and Calbindin in the chick cerebellar cortex. **A:** SV2 could be detected in the inner part of the molecular layer (ML), partially in the Purkinje cell layer (PL) and in the (internal) granular layer (GL). **B:** Calbindin (Calb) was only found in Purkinje cells (PL) and their developing dendritic and axonal processes. **C:** Merged pictures show a partial overlap of immunosignals in the Purkinje cell layer. Neither in the molecular nor in the granule cell layer Calbindin was detectable. **D:** At the end of the embryonic development, SV2 is localized throughout the molecular layer (ML) and the granular layer (GL). Only few spots are found in the Purkinje cell layer (PL). **E:** Calbindin was detectable in all Purkinje cell somata, the developing dendritic tree in the molecular layer (ML). Also the axons going through the granular layer and merging in the white matter (WM) are Calbindin-immunopositive. **F:** Merging of the both images show a clear separation between SV2 and Calbindin, in the layers descript above. **G:** Immunoreactivity for SV2 is found all over the cerebellar cortex, leaving only the Purkinje cell perikarya-immunonegative. **H:** Calbindin is found in the Purkinje cell perikarya and the matured dendritic tree with all their extensions. **I:** Overlayed pictures enhance the impression of dendritic (postsynaptic) Calbindin and axonal (presynaptic) SV2 immunostaining. Scale bars = 100 μm.

Expression of SV2 and GluR 2/3 During Embryonic and Posthatching Development of Cerebellar Cells and Layers

Immunolabeling for the presynaptic marker SV2 and the AMPA glutamate receptor GluR 2/3 was also investigated from embryonic day 16 (E16) (Fig. 2A–C). SV2 labeling was similar as seen in Figure 1 (compare Figs. 1A and 2A). At this stage, GluR 2/3 was present in the Purkinje cell perikarya and in primary dendrites, as well as in the external granular layer, before the final organization of cerebellar cell layering is finished (Fig. 1B,C).

At E19, diffuse SV2 labeling was present in the molecular layer surrounding primary dendrites but extends also further in the molecular layer toward the cerebellar surface. In the granular layer, SV2 staining began to concentrate in spots (Fig. 2D,F). At this stage, Purkinje cell perikarya and primary dendrites showed well-defined immunostaining for GluR 2/3. In addition, faint immunofluorescence was also detected in the granular layer (Fig. 2E,F).

The basic immunolabeling pattern in the Purkinje cell perikarya and primary dendrites for GluR 2/3 persisted also in the early posthatching days. SV2 labeling in the granular layer was restricted to spots with sharp edges and throughout the molecular layer. This staining pattern remained the same until P20 (Fig. 2G,I). During this time, the signal for GluR 2/3 extended from the cell perikarya to the dendritic tree of the Purkinje cells (Fig. 2H,I).

Higher magnification indicated that SV2 and GluR 2/3 were not colocalized, neither in nor around Purkinje cell perikarya and their dendrites. Figure 3A shows a typical GluR 2/3-positive P10 old Purkinje cell surrounded by SV2-positive spots. At P10, the GluR 2/3-positive perikarya and the dendritic stem of the Purkinje cells were outlined with SV2 immunostaining. At P20 (Fig. 3B), this staining pattern with SV2-positive puncta adjacent to the Purkinje cell perikarya was less prominent. At this stage, the immunostaining was more concentrated around the distal part of the Purkinje cell dendritic tree (compare Figs. 3A and B).

Expression of SV2 and VGlut2 During Cerebellar Development

At E19, SV2 expression is found in spots around Purkinje cells but also clustered in the granule cell layer as well as in the developing molecular layer (Fig. 4A,C). VGlut2, a specific marker for climbing fibers in the cerebellum, was found in the same areas as SV2; however, we detected just a partial overlap between the two proteins (Fig. 4B,C). The clusters in the granule cell layer are mainly double labeled, while immunoreactivity in the molecular layer and surrounding the Purkinje cells is rarely colocalized at this stage (Fig. 4C).

At P10, the colocalization of both proteins has increased. The terminals around the Purkinje cell bodies as well as synapses surrounding the dendritic tree are labeled for SV2 (Fig. 4D,F) and VGlut2 (Fig. 4E,F). Still there are a few terminals in the molecular layer that contain only SV2; few others contain only VGlut2 (Fig. 4E,F).

DISCUSSION

Data about the developmental expression of the presynaptic protein SV2 in the central nervous system are limited. Determining when this protein is expressed by developing cerebellar cells and whether or not it is inserted into cerebellar synapses may help to elucidate the function of SV2 in synaptogenesis of the cerebellum. Here we compared the expression of SV2 with Calbindin and the AMPA glutamate receptor subunit GluR2/3 as postsynaptic markers as well as the vesicular glutamate transporter VGlut2 as a presynaptic marker for climbing fibers.

Changes in the Developmental Distribution of SV2

The embryonic and posthatching development of the chick cerebellum revealed that the expression pattern of SV2 protein is closely linked to the ongoing maturation of Purkinje cells. The SV2 immunoreaction product shifted from the inner part of the molecular layer at E16 steadily toward the cerebellar surface at P20, suggesting an early maturation of synapses between climbing fibers and Purkinje cell dendrites. These data from the chick cerebellum are in agreement with results showing that established synapses between Purkinje cell dendrites and climbing fibers are found already at E19 in the rat (Lachamp et al., 2005; Sugihara, 2006). Immunoreactivity shown for SV2 in climbing fibers followed the maturation of dendrites and reflects dendritic arbor growth of Purkinje cells. At E16, immunoreactivity for SV2 was found in the developing molecular layer as well as in the granular layer, but not in the external granular layer represented by GluR2/3-immunopositive displaced granule cells.

In the early posthatching period, SV2 immunofluorescence was found stronger around Purkinje cell perikarya reflecting nested-type climbing fibers, that develop in the second postnatal week (Sugihara, 2005). We cannot exclude, that the onset of inhibitory basket cells (Weisheit et al., 2006) and their establishment of functional synapses on Purkinje cell perikarya at that time (Zhang and Goldman, 1996; Pouzat and Hestrin, 1997) gives additional immunoreactivity. However, at later stages only few immunopositive puncta were observed around Purkinje cell perikarya due to basket cell terminals, while climbing fiber terminals are now located more in the outer molecular layer. Prominent immunofluorescence for SV2 detected around the distal dendrites includes both, the functional contacts of climbing fibers and the arrival of granule cell parallel fibers around postnatal day 12 in the outer molecular layer (Shimono et al., 1976). Thus, the SV2 immunoreactivity in the chick cerebellum perfectly reflects the development of Purkinje cell perikarya and dendritic connection as well as the remodeling of synaptic contacts as seen in the postnatal development of other species like rats and mice.

Localization of SV2 and GluR 2/3 in Developing Cerebellar Synapses

At E16, presynaptic SV2 immunoreactivity was detected in the inner molecular layer and in the granular layer, suggesting developing climbing fibers that express

this protein. Using double immunofluorescence labeling experiments we found that AMPA receptor subunits 2/3 was expressed in Purkinje cell somata and in their apical dendrites early after they are formed into a single layer at this stage. Over time, the location of SV2 immunolabeling tends to follow the expression pattern of AMPA receptors. In addition, the granular layer exhibits a similar expression pattern of GluR 2/3-positive granule cell dendrites in close apposition to SV2-immunopositive puncta, presumably mossy fiber terminals in glomeruli.

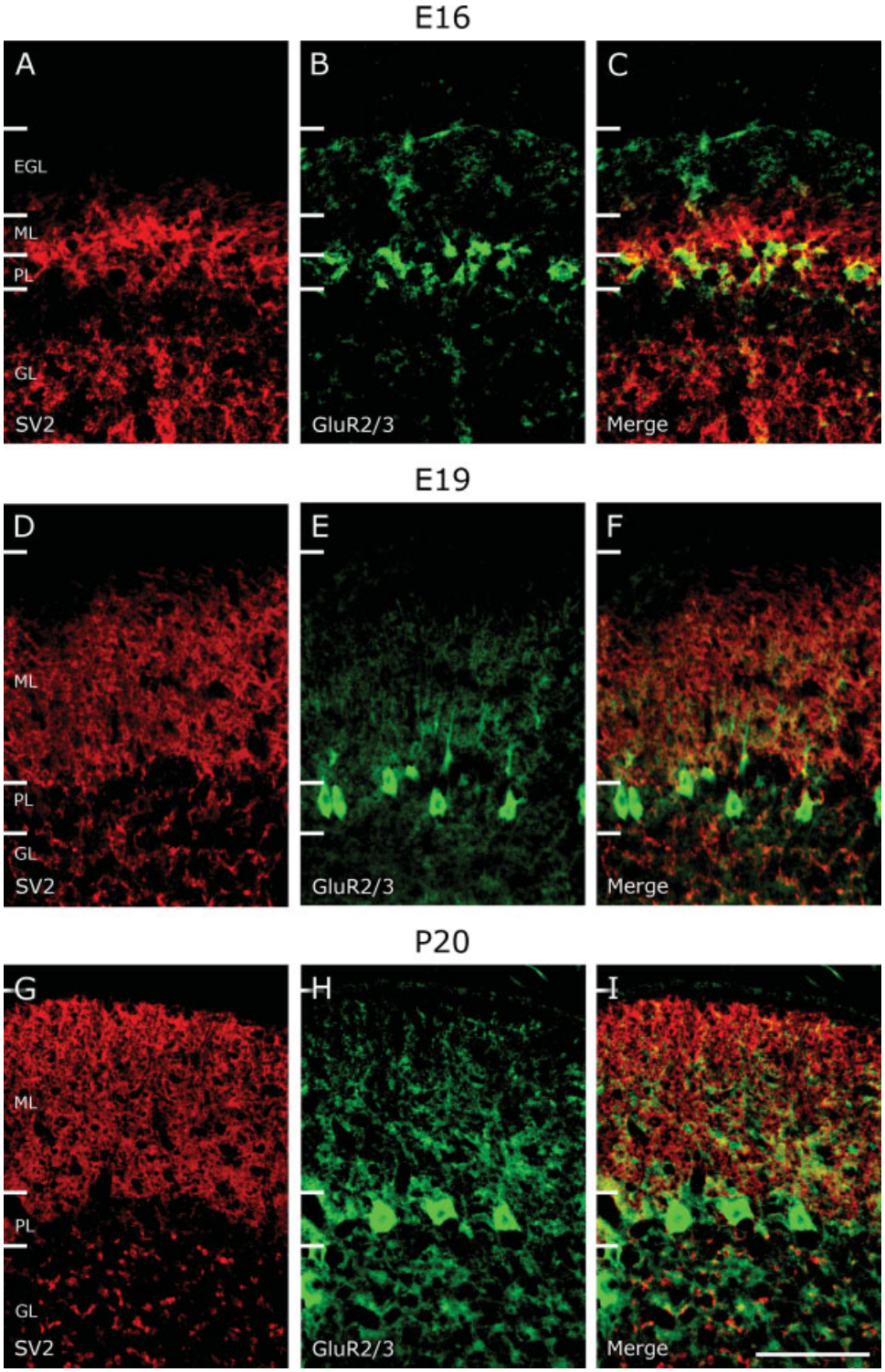

Figure 2.

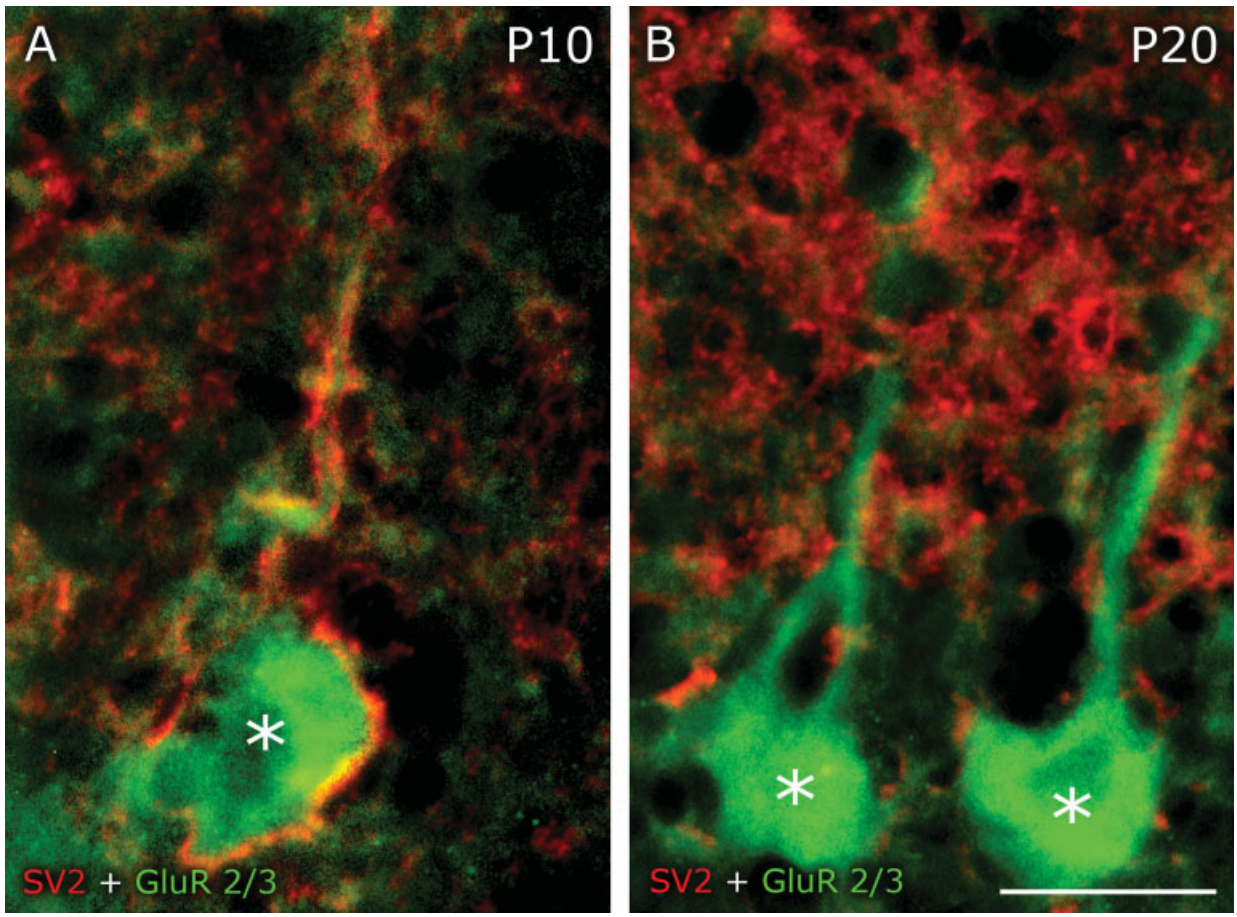

Fig. 3. Expression of synaptic vesicle protein 2 (SV2) and glutamate receptor subunits 2/3 (GluR 2/3) in the chick Purkinje cell layer. Merged double immunofluorescence showed that GluR 2/3 was found mainly in Purkinje cell bodies and dendrites. Immunostaining for SV2 was found in the Purkinje cell layer and molecular layer, but not in Purkinje cells and dendrites itself. Immunopositive puncta and lines surround Purkinje cell perikarya and dendrites. **A,B:** At posthatching day (P) 10 (A), more and stronger immunofluorescence was found around cell perikarya, while at stage P20 (B) only few immunopositive puncta were observed around Purkinje cell perikarya, and immunofluorescence for SV2 was stronger around the dendrites in the molecular layer. Scale bar = 25 μm.

This early synaptic appearance of both pre- and postsynaptic proteins in the developing chick cerebellum is similar to other species (Petralia and Wenthold, 1992; Bergmann et al., 1996; Lachamp et al., 2005). This finding supports the idea of early synaptogenesis between developing axons (from olivar origin; Palacios-Pru et al.,

Fig. 2. Expression of synaptic vesicle protein 2 (SV2) and glutamate receptor subunits 2/3 (GluR 2/3) in the chick cerebellar cortex. **A:** No immunofluorescence for SV2 was detected in Purkinje cells at this developmental stage. SV2 was found apart from Purkinje cell perikarya in the adjacent molecular layer, where Purkinje cell primary dendrites develop. The internal granular layer exhibited a patchy expression pattern for SV2, while no SV2 immunofluorescence was found in the external granular layer. **B:** Immunofluorescence for GluR 2/3 was found mainly in the developing Purkinje cell layer. The somata of the Purkinje cells were GluR 2/3-positive. In addition, faint staining was seen in the internal and external granular layer. **C:** The merged pictures show that SV2 was localized around the GluR 2/3-positive Purkinje cell perikarya and the dendritic stem, while neither in the outer part of the molecular layer nor in the granular layers immunostaining was seen for SV2. **D:** With ongoing development of the Purkinje cell dendritic tree, clear immunofluorescence for SV2 was found in the developing molecular layer. **E:** The somata of the Purkinje cells and their developing primary dendrites in the inner molecular layer were GluR 2/3-positive. **F:** Prominent immunoreactivity for GluR 2/3 was found in Purkinje cell perikarya and their developing dendritic tree, whereas SV2 labeling was now distributed over the molecular layer. In the granular layer, a patchy pattern of GluR 2/3, intermingled by SV2-positive puncta was seen in this embryonic stage. **G:** Diffuse SV2 immunostaining was found in the entire molecular layer, while maximal immunofluorescence for SV 2 was found in the outer part of the molecular layer. In posthatched stages, the granular layer showed a dotted expression pattern for SV2. **H:** Immunofluorescence for GluR 2/3 was found primarily localized to Purkinje cell somata and the dendritic stem in the molecular layer. In addition, diffuse immunostaining was seen in the granular layer. **I:** Merged pictures exhibit better how SV2-positive puncta and lines surround Purkinje cells. In the molecular layer, immunostaining outlines the dendritic tree. Immunostaining was stronger in the outer molecular layer and declines toward the deep molecular layer. In the granular layer, a patchy pattern of GluR 2/3 immunoreactivity, interspersed with and surrounded by SV2-positive puncta was detected. Scale bars = 100 μm.

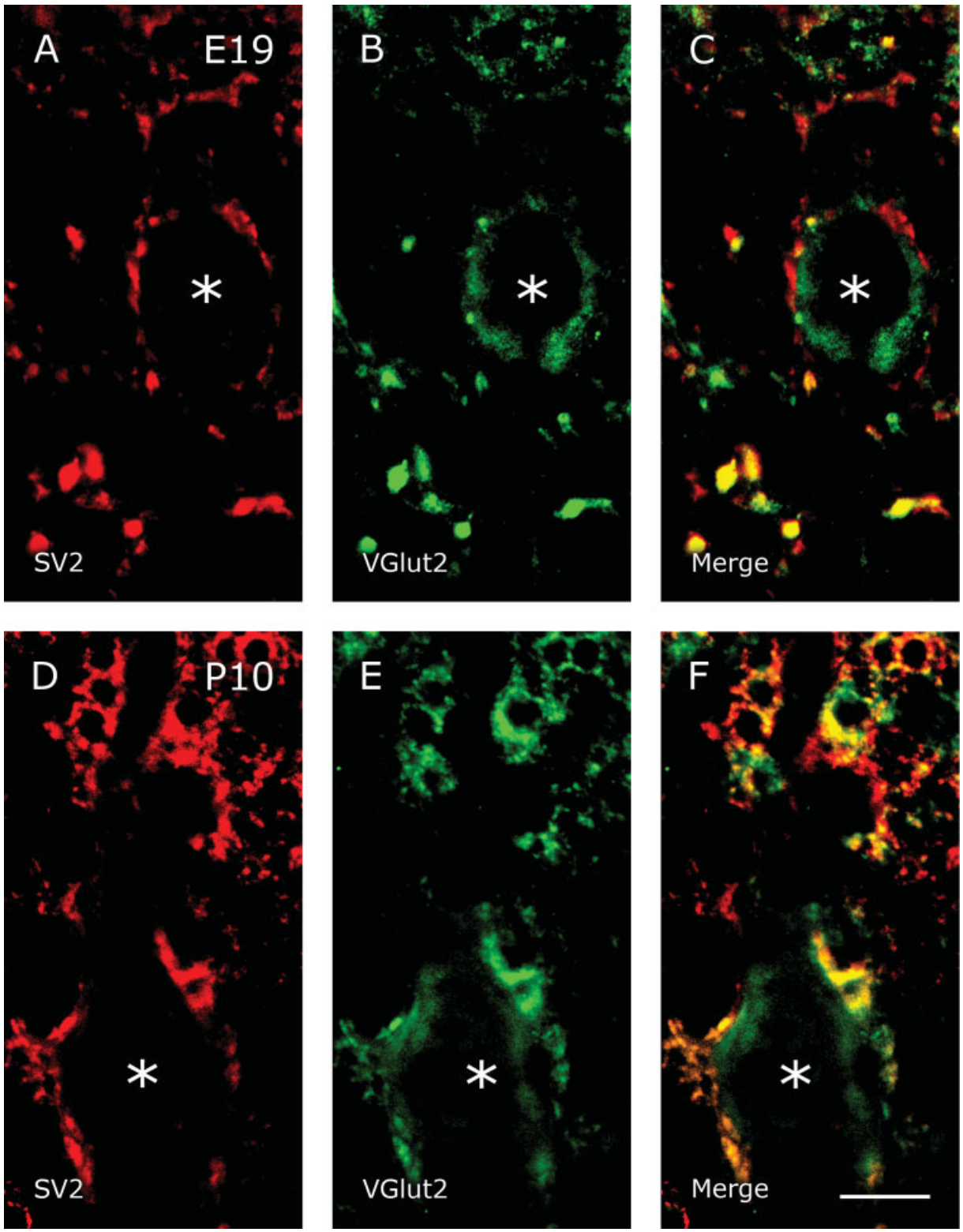

Fig. 4. Expression of synaptic vesicle protein 2 (SV2) and vesicular glutamate transporter 2 (VGlut2) in the chick cerebellum. **A:** SV2 was found outside of the Purkinje cell perikarya (star) as well as in spots localized in the molecular layer (ML) and the granular layer (GL). **B:** Diffuse immunosignals of VGlut2 were found in the Purkinje cell perikarya (star) and in the molecular layer. Spots in the GL were heavily stained for VGlut2. **C:** Only partial overlap of the immunoreactivity between SV2 and VGlut2 could be detected at embryonic day (E) 19. Strongest overlap was found in the GL spots. **D:** SV2 staining was now much stronger in the ML and persisted around the Purkinje cell perikarya. **E:** At postnatal stage 10, VGlu2 was almost colocalized with SV2. However, few spots in the ML are only immunopositive for SV2 or for VGlut2. Scale bar = 25 μm.

1981; Lopez-Roman and Armengol, 1996) and apical Purkinje cell dendrites outlined by AMPA receptors (Pires et al., 2006). Moreover, since it was shown that rat climbing fibers were competent to elicit postsynaptic responses as early as P0 and, because they express some important presynaptic proteins (VGlut, Lachamp et al., 2005 and SV2 this study), these data suggest that the established synapses are competent for exo- and endocytosis, which was described earlier also for hippocampal neurons (Matteoli et al., 1992; Kraszewski et al., 1995; Grosse et al., 1998).

Recent data implicated that glutamate neurotransmitter mediates not only excitation in the postsynaptic compartment but modulates also presynaptic function. The colocalization of GluR 2/3 and the presynaptic markers GAD65 or synaptophysin in basket cell axon terminals at Purkinje cell somata support this interpretation of presynaptic AMPA receptors in the cerebellum (Satake et al., 2006). The obtained expression pattern of postsynaptic GluR 2/3 immunoreactivity in Purkinje and granule cell dendrites was present from embryonic stages on and can be detected throughout all posthatched stages. It is in agreement with previous studies suggesting that these principal cerebellar neurons (Purkinje and granule cells) express GluR 2/3 at the postsynaptic site (Bergmann et al., 1996; Petralia et al., 1998), but no colocalization with presynaptic SV2, predominantly found in mossy, climbing, and parallel fibers, was seen.

The data presented here showing an early synaptic expression of SV2, will provide a better understanding of mechanisms leading to proper synaptic stabilization in the cerebellar cortex. Given the implication of SV2 involved in seizure and epilepsy (Lynch et al., 2004; Gillard et al., 2006), it is tempting to speculate that SV2 is a critical factor for cerebellar development and that impairment of this protein can facilitate the progress of epileptic disorders in the adult cerebellar cortex.

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