Eye opening and PSD95 are required for long-term potentiation in developing superior colliculus

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GluN2A complex is re-3). A fourth MAGUK, synapse-no. 2 707 7) and it has a critical period in which considerable visual vision 3 h after controlled eyelid opening (EO) and the vol. 110 pattern vision 31) in three fundamental respects: These data indicate that young SC LTP arises from PSD95 unsilencing pre-EO period when SAP102 binds mixed GluN2A/GluN2B NMDARs. This is followed, in the SC of the neonatal SC and studied their synaptic currents – synaptic effects in area CA1 of rodent hippocampus and mostly in older animals or with cultured hippocampal slices. In the hippocampus, the synaptic MAGUKs synapse-associated protein 102 (SAP102), PSD95, and postsynaptic density protein 93 (PSD93) bind ionotropic glutamate receptors and many molecules through which the receptors signal (1–3). A fourth MAGUK, synapse-associated protein 97 (SAP97), binds selectively to AMPA receptor (AMPA) subunit GluR1 helping to deliver it to, but not remain at the PSD (4).

Although the hippocampus is a highly evolved and important brain region, it is unlikely to reflect properties of all other regions that are critical to brain function. Synaptic development has been studied much more intensively in the visual pathway where it differs significantly from the hippocampus because the onset of pattern vision is necessary for completion of its synaptic connectivity (5–7) and it has a critical period in which considerable visual plasticity disappears (8). Moreover, the superficial superior colliculus (SC), central to eye movements and integration of multiple sensory pathways for orientation in space, is the only brainstem region where synaptic MAGUK function has been studied at all, and it is a region where SAP102 and PSD95 are the only significant MAGUK glutamate receptor scaffolds.

In rodent visual pathways, SAP102 is the dominant scaffold at the PSD until 2–3 h after controlled eyelid opening (EO) and the onset of pattern vision. At this time, PSD95 in visual synapses increases twofold to threefold (9, 10). This is followed, in the SC where retinal, visual cortical, and thalamic inputs converge, by an increase in excitatory synapses (7); a functional refinement of innervating axons (11); an anatomical refinement of the cortico-collicular projection (7); and maturation of SC inhibition (12, 13). Furthermore, in PSD95 KO mice (14), the normal increase in synapse number after EO fails to occur in dorsally oriented vertical neurons of the SC (7).

It is significant that MAGUKs show different expression patterns at different ages because there is increasing evidence that each may bind different signaling molecules at glutamate synapses (15). For example, in rodent hippocampus, SAP102 is present in the early postnatal PSD, it binds NMDA receptors (NMDARs) via GluN2B, AMPARs via stargazin (16), and a complex containing SynGAP (17, 18). In visual cortex, SAP102 is replaced at the PSD by the PSD95 complex containing GluN2A and TrkB upon EO (9, 18). At this stage, SAP102 and GluN2B-rich NMDARs remain in extrasynaptic regions (4, 13) where they mediate evoked currents, whereas the PSD95–GluN2A complex is responsible for immature NMDAR currents (mNMDARs). This scenario, first suggested in the hippocampus (19), was documented in the SC of the developing GluN2A KO mouse (20). Extrasynaptic NMDARs with functions that differ from PSD NMDARs have been identified in several brain regions (13, 21–24) and PSD95 selectivity for the GluN2A tail has now been verified in the developing hippocampus (25).

For NMDARs in forebrain and dorsal midbrain regions, the change from binding MAGUKs via GluN2B to binding via GluN2A has frequently been interpreted as a switch from GluN1/GluN2B diheteromeric receptor currents to short decay-time currents characteristic of GluN1/GluN2A diheteromeric receptors. However, in the SC of rats and mice when SAP102 is still the major PSD MAGUK but GluN2B subunits are increasing, NMDAR decay times decrease abruptly. This is mediated via a calcineurin (CaN)-dependent dephosphorylation of the GluN2A tail (26, 27). Therefore, NMDAR decay time cannot reveal when a subunit change at the PSD from GluN2B/GluN2A to GluN2A occurs or whether SAP102 or PSD95 scaffolds the receptor.

Here, we used short hairpin RNA (shRNA) to KD PSD95 in single cells of the neonatal SC and studied their synaptic currents and NMDAR-dependent plasticity at intervals after EO. Our findings differ from those expected from PSD95 KD experiments in hippocampus CA1 (25, 28–31) in three fundamental respects: In SC, long-term potentiation (LTP) does not survive PSD95 KD; in SC, long-term depression (LTD) does survive PSD95 KD; in SC, reductions in AMPAR and NMDAR synaptic responses occur with PSD95 KD without any change in presynaptic release. In addition, in young SC, the ability to induce LTP is not stable; eyelid reclosure (ERC) for several days causes SC LTP to disappear and eye reopening (ERO) following ERC reinduces LTP. Finally, the CaN-mediated decrease in NMDAR decay times, observed in normal SC neurons only as activity increases before EO (26), is present after EO in PSD95 KD neurons. This suggests that SAP102 binding triheteromeric GluN1/GluN2B/GluN2A NMDARs remains at the PSD in these cells.
Results

LTP Is Labile in the Young SC. In SC, acute slices from postnatal day 15 (P15) to P17 rat pups with EO at P13 or P14, stimulating the stratum opticum at 20 Hz for 20 s produces an NMDAR- and L-type Ca\textsuperscript{2+} channel-dependent LTP in a major excitatory SC neuron population, narrow field vertical (NFV) neurons: a group we have focused LTP studies on because they are visually driven, abundant, and of relatively uniform size (32). This same stimulation applied to slices from pups of the same age with eye closure (EC) showed no LTP in NFV neurons. However, if similarly deprived pups received 4–5 h of visual experience after simultaneous EO, LTP could be induced (Fig. 1A). The same EO-dependent LTP was induced in slices from pups killed 2–4 d after EO, but not in pups with EC during the same period (Fig. 1B). Also when only one eye remained shut and the other was opened at P13 with patterns of visual experience for 3–4 h before killing, LTP was induced only in neurons that had received input from the contralateral open eye (Fig. 1C). In a final paradigm, glued eyelids were opened at P13, and then reclosed on P16. At P20, one-half of the animals had their eyelids reopened for 4–5 h before killing and recording. SC LTP was obtained in slices from these re-opened animals. However, LTP was not present in the reclosed animals whose eyes were not reopened before P20 recording (Fig. 1D). Therefore, young SC LTP had a pronounced dependence on pattern vision as well as a pronounced liability to loss of pattern vision. Importantly, these EC and EO regimes were identical to ones used in the initial study of EC- and EO-associated changes in synaptic levels of PSD95 in visual cortex and SC synapses (9). This tight correlation suggested that PSD95, or molecules in a complex with PSD95 at the synapse were necessary for induction of SC LTP.

SAP102 and PSD95 Are the Only MAGUKs in Young SC. We documented the MAGUKs present in the SC with quantitative Western blotting of PSD95, PSD93, and SAP102. SAP97 immunoreactivity is not present in the SC (33). Homogenates from the SC at P15 and hippocampus at P19, two roughly corresponding stages of synapse maturity (4, 9, 25), were analyzed. Identical protein concentrations from SC and hippocampus were run in adjacent lanes (Fig. 2A), blotted, and probed with antibodies for PSD95, SAP102, and PSD93. SAP102 and PSD95 bands were present in both lanes, with the expected, higher levels in the hippocampus. However, PSD93 was present as an intense band only in hippocampal lamens and barely detectable SC lanes (Fig. 2B).

PSD95 KD Decreases mAMPARc and mNMDARc Frequency. We designed two KD shRNAs (KD I and II) against PSD95, and two scrambled shRNAs (Scr I and II) as negative controls. These shRNAs were inserted into the lentiviral plasmid carrying GFP, and lentiviruses were produced. Efficacy and specificity of these shRNAs were documented in HEK cells and cultured occipital cortical neurons (Fig. 2 B and C). The lentiviruses were injected into the SC of neonates (P1–P3). The eyes of these pups were opened at P14 and they were killed between P15 and P17 for slice physiology with the investigator blind to the shRNA lentiviruses injected. In acute SC slices, infected neurons were identified by their GFP fluorescence followed by differential interference contrast (DIC) microscopy before the slice was fixed for Western analysis (Fig. 2 D). In all cases, PPRs were identical for PSD95 KD and Scr neurons (Fig. 4J). A decrease in both mAMPARcs and mNMDARcs were also unchanged between PSD95 KD, Scr, and uninfected neurons (Fig. 4 S2 D and E, and I and J). Decreases in mAMPARc frequency have been documented in virtually all PSD95 KD or KO studies in CA1 pyramids where they were examined (28–30); however, none of these studies reported changes in mNMDARcs. In the relatively small NFV neurons, it was possible to record mNMDARcs, and similar to the mAMPARcs, their frequencies (Fig. 4 A–D) but not their amplitudes (Fig. S3) were significantly reduced in all PSD95 KD cells. To assay for an effect of PSD95 KD shRNA on presynaptic release (34) that might cause a change in both mAMPARcs and mNMDARcs frequencies, we examined paired-pulse ratios (PPRs). In all cases, PPRs were identical for PSD95 KD and Scr neurons (Fig. 4 E and F). Also consistent with decreases in both mAMPARcs and mNMDARcs frequencies, we found no differences in evoked AMPAR current (eAMPARc)/evoked NMDAR current (eNMDARc) ratios between neurons expressing KD I or II and their corresponding Scr controls (Fig. 4 G and H).

In Vivo PSD95 KD Eliminates NMDAR-Dependent LTP. Characterization of SC LTP in whole-cell patch-clamped NFV neurons expressing the PSD95 KD or the corresponding Scr shRNA revealed that LTP was absent in all PSD95 KD neurons but present in all Scr-expressing neurons (Fig. 5 A and B). In addition, because the mAMPARc and mNMDARc recordings indicated reduced numbers of AMPAR- and NMDAR-containing...
synapses in PSD95 KD cells, we compared the amplitude and frequency of responses to inducing stimuli that were determined for each neuron as the intensity producing a response of half-maximal size. We found no differences in the amplitudes of the evoked responses or in their ability to follow each stimulating pulse between neurons expressing the PSD95 KD or corresponding Scr shRNAs (Fig. S4).

**LTD Is Normal in SC PSD95 KD Neurons.** Colledge et al. (35) proposed that PSD95 removal could be causative in NMDAR-dependent LTD. Also, LTD is absent in the hippocampus of PSD95 KO mice (14, 15, 28) and impaired in PSD95 KD neurons in cultured hippocampal slices (30, 31). However, there is evidence that only extrasynaptic NMDARs are coupled to LTD generation (36, 37), and in the SC, normal NMDAR-dependent LTD is present in young GluN2A KO mice (38). These mice have no mNMDARcs and spontaneous NMDAR currents (sNMDARcs) probably because once PSD95 is at the PSD, it requires the GluN2A tail to bind NMDARs (13, 20). Moreover, in both WT and GluN2A KO mice, SC LTD can be eliminated by blockade of either GluN2B receptors or L-type Ca2+ channels (38). Consequently, we hypothesized that NMDAR-dependent LTD in the PSD95 KD SC neurons would not show disrupted LTD. Indeed, we found that NMDAR-dependent LTD remained in these neurons and was identical in amplitude to the LTD induced in neurons expressing the corresponding Scr shRNA (Fig. 5 C and D).

Fig. 2. PSD95 and SAP102 dominate in the young SC, and shRNA against PSD95 causes effective KD of this MAGUK in HEK cells and cultured cortical neurons. (A) Homogenates from rat SC and hippocampus (Hip) at P15 and P19, respectively, analyzed by Western blotting (20 μg lane). SAP102 and PSD95, but not PSD93, are the two dominant MAGUKs expressed in the young SC. Compared with corresponding protein levels in Hip, SAP102 and PSD95 were significantly lower and PSD93 was very significantly lower in SC. (B) HEK 293 cells were cotransfected with PSD95-GFP and either a PSD95 KD shRNA plasmid or a scrambled (Scr) shRNA plasmid. After 48 h of cotransfection, cell lysates were analyzed by Western blotting. Both PSD95 shRNA KD I and KD II eliminated detectable PSD95, whereas shRNA Scr I and Scr II had no effect on PSD95 expression. (C) Cultured occipital cortical neurons were infected with PSD95 KD or scrambled shRNA lentiviruses at day in vitro (DIV) 2 and analyzed by Western blotting at DIV 21. Endogenous protein expression level of PSD95 was reduced by PSD95 shRNA KD I or KD II, whereas shRNA Scr I or Scr II did not change PSD95 expression.

**FK506 Increases sNMDAR Decay Time in PSD95 KD Neurons.** After PSD95 KD, the only other significant MAGUK in the young SC, SAP102, was expected to be the remaining scaffold at the PSD. However, in all of the PSD95 KD cells we recorded sNMDARcs with the short decay-time characteristic of the diheteromeric GluN1/GluN2A NMDARs usually bound by PSD95 after EO (9) in both the PSD95 KD and Scr neurons (Fig. 6 A, C, E, and G, upper three traces). In normal SC neurons, the effect of the CaN blockade disappears before EO as the level of PSD95 and GluN2A increases (9, 12). However, the GluN2A subunit protein, first detectable in the SC at ~P7, is significantly increased in the P15–P17 EO pups studied here (12). It is also likely that these increases are independent of PSD95. Consequently, we tested the hypothesis that pre-EO decrease in NMDARc decay times that were observed with GluN1/GluN2B and GluN2A/GluN2B heteromeric NMDARs bound by SAP102 had reappeared in PSD95 KD neurons by applying the membrane-permeable CaN antagonist FK506. In the study by Shi et al. (26), both FK506 and the CaN inhibitory peptide effectively eliminated the decay-time decrease. We bath-applied FK506 and within 12 min PSD95 KD neurons developed long sNMDARc decay times (Fig. 6 C and G, lower three traces, and D and H) typical of GluN2A/GluN2B-rich NMDARcs in younger animals with SAP102 as the PSD MAGUK. FK506 had no effect on neurons carrying the corresponding Scr shRNA (Fig. 6 A and E, lower three traces, and B and F). The results indicate that SAP102 binding NMDARs by the GluN2B C-terminal (16) supported GluN2B/GluN2A NMDARs at the PSD despite EO, and that the generally higher levels of activity after EO when it impinge on the PSD95 KD neurons triggered the same activity-dependent CaN response documented by Shi et al. (26). Thus, the normal NMDAR activity-dependent CaN shortening of NMDARc decay times found before EO could remain active on triheteromeric GluN1/GluN2A/GluN2B NMDARs at older SC synapses because activity was sufficiently high and because SAP102 binding NMDARs by the GluN2B C-terminal remained at the PSD.

**Discussion**

Despite many reports describing the functions of PSD95 at glutamate synapses, identification of specific roles for synaptic MAGUKs has been difficult because the KD and KO work has been focused on the hippocampus where four MAGUK family
In vivo PSD95 KD affects LTP, but not LTD. (\(P = 0.43\]. The black bars \(P < 0.05\), \(P = 0.01\); KD I: 0.99 \(P = 0.04\), \(n = 0.04\), \(n = 0.04\), \(n = 0.03\); KD II: 0.74 \(P < 0.02\). (9/3, vs. KD II: 0.93 \(P < 0.02\)) The bar graphs showing significant differences in mNMDARc frequency \((E)\) shows corresponding decreases and reincreases of PSD95 staining in SC synaptic increases is fully consistent with the study by Phillips et al. Therefore reinforce the present results by showing that new SC synapses resulting from activity increases cannot be stabilized unless PSD95 is present. This report shows that SC LTP disappears with several days of ERC after EO, and reappears with ERO. This is completely consistent with the data of Yoshi et al. (9) showing corresponding decreases and reincreases of PSD95 levels in visual synapses using the same EO, EC paradigms used in SC. Furthermore, the finding that mixed triheteromeric NMDARs reappear at the PSD of PSD95 KD cells supports our previous proposal that the insertion of PSD95 actively displaces SAP102 bound receptors from the center to the extrasynaptic region of synapse (13). The current evidence for a crucial function of PSD95 in SC synaptic increases is fully consistent with the study by Phillips et al. (7) where another type of SC neurons, dorsally oriented vertical neurons, in the PSD95 KO mouse (14) fail to show the significant increase in synapse number found in WT SC neurons upon EO and the onset of pattern vision. These new synapses are from the cortico-collicular projection, which develops later than retinal inputs, and, without EO, this set of converging inputs is not only functionally but also structurally withdrawn (7). The data of Phillips et al. therefore reinforce the present results by showing that new SC synapses resulting from activity increases cannot be stabilized unless PSD95 is present. This report shows that SC LTP disappears with several days of ERC after EO, and reappears with ERO. This is completely consistent with the data of Yoshi et al. (9) showing corresponding decreases and reincreases of PSD95 levels in visual synapses using the same EO, EC paradigms used in SC.

In acute SC slices, robust LTP requires NMDAR and L-type Ca\(^{2+}\) channel activity (32) as well as GluN2A subunits at the PSD (20, 38), and, as shown here (Fig. 1), SC LTP in the superficial visual layers only occurs after EO. This potentiation results almost entirely from un silencing of silent synapses (32). Fig. 4A and B demonstrates that PSD95 KD eliminates SC LTP; therefore, both high levels of synaptic PSD95 and its unique ability to bind NMDARs having two GluN2A subunits at the center of SC synapses are necessary for NMDAR-dependent LTP in the developing SC. Furthermore, the finding that mixed triheteromeric NMDARs reappear at the PSD of PSD95 KD cells supports our previous proposal that the insertion of PSD95 actively displaces SAP102 bound receptors from the center to the extrasynaptic region of synapse (13). The current evidence for a crucial function of PSD95 in SC synaptic increases is fully consistent with the study by Phillips et al. (7) where another type of SC neurons, dorsally oriented vertical neurons, in the PSD95 KO mouse (14) fail to show the significant increase in synapse number found in WT SC neurons upon EO and the onset of pattern vision. These new synapses are from the cortico-collicular projection, which develops later than retinal inputs, and, without EO, this set of converging inputs is not only functionally but also structurally withdrawn (7). The data of Phillips et al. therefore reinforce the present results by showing that new SC synapses resulting from activity increases cannot be stabilized unless PSD95 is present. This report shows that SC LTP disappears with several days of ERC after EO, and reappears with ERO. This is completely consistent with the data of Yoshi et al. (9) showing corresponding decreases and reincreases of PSD95 levels in visual synapses using the same EO, EC paradigms used in SC.
here. It remains to be seen whether this highly labile form of synapse potentiation and stabilization is also present in older brains and other visual centers after prolonged pattern vision deprivation.

**LTD Survives PSD95 KD in SC.** This finding also differs from those in the hippocampus where the KD or KO of PSD95 eliminates NMDAR-dependent LTD. In the hippocampus where PSD95 remains to compensate for PSD95 KO or KD (14, 15, 30, 31), LTD but not LTP disappears. Carlisle et al. (15) suggest that PSD95 may bind the signaling complex necessary for LTD while PSD95 is involved in LTD and normally mitigates the potentiating effects of LTP resulting in the enhanced LTD seen in the hippocampus of genetic PSD95 KO rats (14, 28). However, the lack of PSD93 in the young SC is not consistent either with the loss of LTD or the maintenance of LTD in this structure when PSD95 is depleted. Xu et al. (31) also provided evidence for PSD95 involvement in LTD. With a series of deletion constructs and point mutations in the PSD95 C terminus following WT PSD95 depletion by shRNA, they showed that the C-terminal domain of PSD95 normally scaffolds the signaling complex necessary for CA1 LTD. This explanation is also not consistent with our finding that NMDAR-dependent LTD is maintained in SC neurons after PSD95 KD. However, CaN activity is believed to be critical to NMDAR-dependent LTD at least in the hippocampus (44, 45), and, as noted above, both GluN2B and L-type Ca\(^{2+}\) channels are necessary for SC LTD. In addition, this study demonstrates CaN involvement in decreasing sNMDARc decay time when SAP102 is the only remaining major MAGUK in SC neurons. These findings suggest that the early appearing MAGUK SAP102 that is still highly expressed in the young SC after EO can also scaffold the complex for inducing LTD.

**CaN-Mediated Decrease of sNMDARc Decay Time.** A final finding in this study is that the CaN-mediated decrease in sNMDARc decay times (26) observed in normal SC neurons can be retained after EO in SC PSD95 KD neurons. This CaN effect involves a dephosphorylation of at least one protein kinase A (PKA) site, serine 900, on the GluN2A cytoplasmic tail (27). Similar CaN activity was described by Lieberman and Moody (46) as a CaN-dependent change in NMDAR channel open time, in analyses of single channel currents. Krupp et al. (47) found that the same decrease in NMDAR decay time resulted from CaN dephosphorylation of two serine sites on the GluN2A cytoplasmic tail. The present data show that this NMDARc decay-time shortening can reappear when PSD95 KD causes the GluN2B subunit composition of NMDARs at PSDs to be unusually high and when input activity is increased due to pattern vision. The finding is potentially significant for understanding differences between the subunit makeup of NMDARs throughout the brain and the activity-dependent control of the receptor’s current. For example, Flint et al. (48) documented short NMDARc decay times characteristic of GluN1/GluN2B diheteromeric NMDAR in young somatosensory neurons when measured levels of GluN2A were still extremely low. They concluded that just one GluN2A subunit in a triheteromeric receptor with GluN2B was sufficient to shorten the decay times of the NMDARs. It is likely that the CaN-mediated dephosphorylation of GluN2A is responsible for the short NMDARc decay times reported by Flint et al. (48). The same CaN mechanism may also explain why recent biochemical analyses of synaptic NMDAR composition report a prevalence of GluN1/GluN2B/GluN2A triheteromers at mature glutamate synapses even though sNMDARc decay times are short in the mature brain (49, 50). Finally, the reappearance of this CaN effect in older SC neurons when levels of triheteromeric NMDARs are abnormally present and when converging glutamatergic activity is high suggests that it may represent yet one more mechanism for homeostatically regulating cytoplasmic Ca\(^{2+}\) concentrations in the brain (51).

**Conclusion**

We document a requirement for PSD95 for the maintenance of the normal levels of mAMPARs and mNMDARs frequency, normal synaptic input, and for the appearance of NMDAR-dependent LTP in the SC where only SAP102 and PSD95 MAGUKs are normally prominent. Unlike normal SC neurons where diheteromeric GluN1/GluN2A NMDARs are at the PSD, in PSD95 KD SC neurons triheteromeric NMDARs bound by SAP102 are at the PSD. Nevertheless, in the P15–P17 rats studied, the SAP102 MAGUK cannot replace the PSD95 function of binding GluN2A diheteromeric receptors or facilitating NMDAR-dependent LTP. Most significantly, this study suggests that much of the intensive and sophisticated work on MAGUK function in hippocampal CA1, may not generalize to synaptogenesis and developmental plasticity in many other regions of the vertebrate central nervous system.

**Methods**

**Animals.** Sprague Dawley rat pups were treated with synchronized EC, EO, ERC, and ERO procedures as described previously (9). All experiments were
performed in accord with the guidelines of the Massachusetts Institute of Technology Institutional Animal Care and Use Committee.

Construction of Lentiviral Vectors. Two shRNAs (shRNA KD I and II) against mRNA sequences unique to PSD95 and also two similar but scrambled shRNAs (shRNA Scr I and II) as negative controls were designed. These were inserted into the lentiviral plasmid carrying GFP, and lentiviruses were produced in HEK cells (see SI Methods for shRNA sequences and further details).

Expression Analysis of MAGUKs in Developing SC and Hippocampus. Homogenates from rat SC and hippocampus at P15 and P19, respectively, were analyzed by Western blotting for evaluation of PSD95, SAP102, and PSD93 protein levels (SI Methods).

Injection of Lentivirus into the SC. P1–P3 rat pups were cold-anesthetized, and lentivirus solution (0.1 μL) was injected into the SC (SI Methods).

Electrophysiological Recordings in Mouse Adult Slices. Acute parasagittal SC slices were prepared as previously described (32). Whole-cell recordings were made in NPY neurons in the mid-stratum griseum superficiale of the SC. Detailed conditions for recordings of excitatory postsynaptic potential, excitatory postsynaptic current, LTP/LTD induction, measurement, and chemicals are described in SI Methods.

Statistics. Data are shown as mean ± SEM, and n is given as the number of neurons recorded/the number of animals used. Statistical significance was determined using the two-tailed unpaired Student t test unless otherwise stated following an F test indicating a normal distribution.

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