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Structural Plasticity of Synaptopodin in the Axon Initial Segment during Visual Cortex Development

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Abstract

The axon initial segment (AIS) is essential for action potential generation. Recently, the AIS was identified as a site of neuronal plasticity. A subpopulation of AIS in cortical principal neurons contains stacks of endoplasmic reticulum (ER) forming the cisternal organelle (CO). The function of this organelle is poorly understood, but roles in local Ca2+ trafficking and AIS plasticity are discussed. To investigate whether the presence and/or the size of COs are linked to the development and maturation of AIS of cortical neurons, we analyzed the relationship between COs and the AIS during visual cortex development under control and visual deprivation conditions. In wildtype mice, immunolabeling for synaptopodin, ankyrin-G, and βIV-spectrin were employed to label COs and the AIS, respectively. Dark rearing resulted in an increase in synaptopodin cluster sizes, suggesting a homeostatic function of the CO in this cellular compartment. In line with this observation, synaptopodin-deficient mice lacking the CO showed AIS shortening in the dark. Collectively, these data demonstrate that the CO is an essential part of the AIS machinery required for AIS plasticity during a critical developmental period of the visual cortex.

Key words: axon initial segment, cisternal organelle, sensory deprivation, synaptopodin, visual cortex

Introduction

The visual cortex (V1) depends on sensory information to remodel its immature and highly plastic circuits into a mature and stable neuronal network during defined critical periods of cortical plasticity (CP) (Katz and Shatz 1996; Hensch 2005). Whereas the importance of somatodendritic plasticity mechanisms have been intensely studied (Levitt and Hubener 2012), little is known about plasticity occurring during the CP in the axon initial segment (AIS), a putative key player in activity-driven neuronal plasticity (reviewed in Yoshimura and Rasband 2014; Adachi et al. 2015). The AIS is an electrogenic and highly specialized axonal compartment mostly located close to the soma (reviewed in Rasband 2010; Leterrier 2016). Formation and maintenance of the AIS require the scurfolding protein Ankyrin-G (ankG) (Rasband 2011; Jenkins et al. 2015), which clusters sodium and potassium channels for action potential (AP) initiation (Kole and Stuart 2012) and links membrane proteins to the periodically arranged actin cytoskeleton (Rasband 2011; D’Este et al. 2015; Leterrier et al. 2015).
In work leading to this study, we reported structural changes of the AIS during the CP depending on visual input (Gutzmann et al. 2014). These changes could be divided into 3 phases, that is, a significant AIS length increase until eye-opening at P10/15 (phase 1), followed by AIS shortening at the peak of the CP (P28, phase 2), and finally re-elongation and stabilization of the AIS from the closure of the CP (P35) onward (phase 3). In cortical neurons of dark-reared mice, AIS maintain their juvenile length distribution and fail to shorten (Gutzmann et al. 2014). Functional studies by others showed that changes in AIS length and distance from the soma are associated with changes in neuronal drive and excitability both in vivo and in vitro, although relocation events of the AIS so far have to be robustly reproduced in vivo (Grubb and Burrone 2010; Kuba et al. 2015; Evans et al. 2015; Wefelmeyer et al. 2015; Guldledge and Bravo 2016): short AIS are the result of an increased synaptic drive and are correlated with reduced neuronal excitability, whereas long AIS result from decreased synaptic drive (e.g., after sensory deprivation) and are correlated with increased neuronal excitability.

AIS plasticity may depend on changes in intraxonial Ca\(^{2+}\) levels, since Ca\(^{2+}\) currents modulate the generation and timing of APs in this compartment (Bender and Trussell 2012). The cisternal organelle (CO), which consists of elongated cisterns of smooth endoplasmic reticulum (sER) held together by electron dense material (Peters et al. 1968; Kosaka 1980; Spacek 1985; Benedeczky et al. 1994), is a putative regulator of AIS Ca\(^{2+}\)-trafficking (Benedeczky et al. 1994; Bas Orth et al. 2007). Formation of the CO depends on the actin-associated protein synaptopodin (synpo), which is also an excellent marker molecule to identify the CO within an AIS (Bas Orth et al. 2007; Sanchez-Ponce et al. 2011). Since location and putative function implicate the CO in the regulation of AIS plasticity, we hypothesized that its presence could influence changes in AIS structure in V1 during normal development and/or under conditions of visual deprivation. Synpo immunolabeling was employed to visualize COs in wildtype mice and synpo-deficient mice used to study AIS maturation in the absence of COs. Our data indicate that the presence of synpo has profound effects on AIS structural maturation during V1 development in particular during the critical period of cortical plasticity (CP) and after loss of visually-driven synaptic input.

**Material and Methods**

**Animals**

Animal experiments were performed in agreement with institutional and national laws. Synpo KO mice (Deller et al. 2003) backcrossed to C57BL/6 for more than 10 generations were obtained from a colony at Goethe University Frankfurt. Heterozygous animals were intercrossed to generate homozygous mice. Animals of mixed gender from the wildtype C57BL/6JR strain (Janvier Labs, France) and synpo KO mice were maintained with food and water ad libitum on a regular 12 h light/dark cycle (except in deprivation studies as outlined below).

**Developmental Study**

For the analysis of visual cortex development, a total of 6 brains were analyzed in each of the following age groups: P7, P10, P12, P15, P21, P28, P35, and adult (P > 55) for wildtype and P1, P7, P10, P15, P21, P28, P35, and adult (P > 55) for synpo KO mice. Over 100 AIS per animal were examined in the primary visual cortex (V1) in layers II/III and V, respectively. For the analysis of a nonsensory cortex control, AIS length of layer II/III was measured in the cingulate cortex. A total of 3 brains from P10, P15, P28 (control), P > 55 as well as P0–28 (dark) were used. A minimum of 50 AIS per animal were analyzed.

**Visual Deprivation**

Wildtype and synpo KO mice of various ages were kept in dark cages with food and water ad libitum. Total absence of light was controlled for by exposure of photographic paper located in the cages. Groups of 6 mice each were reared in complete darkness from P0–21, P0–28, P0–35 and kept in darkness from P60–67. Reversal experiments were performed by exposing P28 dark-reared mice to light for 1 week until P35. Analyses were performed immediately after the visual deprivation period. Data from the present developmental study served as controls for all visual deprivation experiments. Experimental groups are summarized in Table 1.

**Immunofluorescence**

Immunofluorescence was used to analyze the development of synpo expression and AIS maturation in age groups of wildtype and synpo KO mice ranging from P1 to P > 55 (adult). P1–P7 animals were decapitated and brains were dissected in ice-cold 0.1 M phosphate buffer (PBS), fixed overnight by immersion in 1% paraformaldehyde (PFA, in 0.1 M PBS, pH 7.4) at 4 °C and cryoprotected in 10% sucrose followed by 30% sucrose (overnight) at 4 °C. Animals P10 and older were exsanguinated with 0.9% NaCl under deep anesthesia with Ketamine (120 mg/kg BW)/Xylazine (16 mg/kg BW) and perfusion-fixed with ice-cold 1% PFA.

Table 1: Synopsis of experimental and control groups

| Mouse strain | Control | Visual deprivation | Fixation (1% PFA) |
|--------------|---------|-------------------|------------------|
| Wildtype C57Bl/6JR (n = 6) | P1 | From birth (P0–21) | Immersion |
| | P10, P12, P15 | From birth (P0–28) | Perfusion |
| | P21 | From birth (P0–28)/reversal in light (P28-35) | Perfusion |
| | P28 | From birth (P0–35) | Perfusion |
| | P > 55 | From P60–67 | Perfusion |
| Synpo KO 129/C57Bl6 (n = 6) | P1, P7 | From birth (P0–21) | Immersion |
| | P10, P15 | From birth (P0–28) | Perfusion |
| | P21 | From birth (P0–28)/reversal in light (P28-35) | Perfusion |
| | P28 | From birth (P0–35) | Perfusion |
| | P > 55 | From P60–67 | Perfusion |
After overnight immersion in 1% PFA at 4 °C, brains were cryoprotected in 10% sucrose followed by 30% sucrose (overnight) at 4 °C. Tissue was trimmed to a block including V1 and embedded in Tissue Tek (Sakura Finetek). Double and triple immunofluorescence was performed on free-floating 40-μm thick cryostat sections for age groups P10 and older. Sections (20 μm) from younger age groups were stained directly on slides. Immunostaining was performed as described (Gutzmann et al. 2014). After omission of the primary antibodies no specific immunolabeling was observed. Antibodies and protocols used in this study are summarized in Supplementary Materials (Supplementary Table 1).

Confocal Analysis and Image Analysis
Confocal analysis was carried out on a C1 Nikon confocal microscope with a 60x objective (oil immersion, numerical aperture of 1.4) and a Leica SP5 confocal microscope with a 63x objective (oil immersion, numerical aperture of 1.4). To increase the number of in-focus immunoreactive structures, stacks of images were merged (maximum intensity projection). Thickness of single optical sections was 0.5 μm in stacks of 10–20 μm total depth. Confocal x-y-resolution was kept at 0.21 μm per pixel. Images for qualitative analysis were evaluated and enhanced for contrast in Fiji (ImageJ) and Photoshop CS (Adobe Systems). Three-dimensional projections from stacks were processed first by blind iterative deconvolution (theoretical PSF based on the optical properties of the microscope and the sample, 10x iteration) according to standard procedures in AutoQuant × 3 (Media Cybernetics). Subsequently, to visualize x-y-z information about synpo localization within or outside of any given AIS, deconvolved files were reconstructed (surface) using Imaris 8.1.2 (Bitplane).

AIS Length and Synpo Expression Analysis
AIS length was measured using a self-written macro (Gutzmann et al. 2014). For the purposes of this study, additional functions for analyzing number and size of synpo clusters in AIS were added. Analysis was performed as follows: AIS containing synpo-positive immunofluorescence signals were manually outlined. Synpo signals outside AIS—likely located in dendritic spines (Deller et al. 2000)—were eliminated using the “Clear Outside” function of ImageJ. For cluster analysis, the “Color Threshold” option was used. Threshold level was set to 50 and minimum size of pixels was 5. Both values were kept constant during measurements. All objects within these parameters were included by the “Analyze Particle” function in ImageJ. Mean number and size (in pixels) of synpo-positive clusters per AIS were calculated. For cluster sizes, mean pixel values were translated into μm² (area [μm²] = area [pixels] × (microscope resolution)²).

βIV-spectrin fluorescence intensity profiles at the AIS were quantified according to a previously published analysis of ankG expression at the AIS (Tapia et al. 2017). Briefly, fluorescence intensities obtained during the application of the above mentioned AIS length analysis were plotted per pixel (= 0.21 μm) and normalized to the maximum fluorescence intensity value measured in each AIS. Data were smoothed using Sigma Plot 12.5 software (Systat Software GmbH) resulting in average βIV-spectrin fluorescence intensity every 1 μm. The whole length of AIS was taken into account. For each analyzed age and condition (only layer II/III), a minimum number of 100 AIS were measured in 6 animals per age and condition.

Statistical Analysis
Mean values and standard error of the mean (SEM) of AIS length, size, and number of synpo clusters per AIS were calculated using Excel (Microsoft), and plotted and analyzed in GraphPad Prism 7 software (GraphPad Software, Inc.), or Sigma Plot 12.5 software (Systat Software GmbH). Wilcoxon rank-sum test was carried out for comparison of 2 groups. For the statistical comparison of 3 or more groups Kruskal–Wallis test followed by Dunn’s post hoc test was used. For the comparison of AIS length between wild-type and synpo KO mice during development, 2-way ANOVA with Dunn–Šidák correction was applied. For frequency histograms of AIS length bin-centers were classified: The entire range of AIS length values (10–60 μm) was divided into a series of intervals with steps of 2 μm. AIS were then counted and assigned to each interval. To measure how synpo number and synpo size vary with AIS length, age of the animal and condition (control/dark), respectively, Spearman rank correlation was performed (Supplementary Table 3). To predict AIS length from age, condition and cortex layer, a linear multiple regression model (y = x₀ + x₁ + x₂) was applied (Supplementary Table 3). The function describes how the y-variable AIS length relates to the 3 independent x-variables age, condition, and cortical layer.

Results
Synpo Expression in the AIS of V1 Neurons Correlates with the Time of Eye-Opening
The expression of synpo was investigated in AIS of layers II/III and V pyramidal neurons during V1 development in mice. Synpo expression was first detected at P7 (Fig. 1A). At this time point synpo-positive clusters were not co-localized with AIS markers, suggesting that synpo is sorted to dendritic spines before being sorted to the AIS. At P10, co-localization of synpo and βIV-spectrin was first detected and increased until P15 (Fig. 1B, D). Expression of synpo reached a plateau in adult animals (Fig. 1C, D, Supplementary Fig. S1).

Next, we quantified the percentage of synpo-positive AIS in layers II/III and V. In line with our qualitative observations, we found an increase of the percentage of synpo positive AIS from P10 until adulthood (Fig. 1D). Synpo expression in AIS began around P10 (12.8 ± 2.5% for layer II/III, Fig. 1D; 19.2 ± 2.6% for layer V; see Supplementary Fig. S2A; Supplementary Table 2) and increased with time. At P15, the percentage of synpo-positive AIS was highest in layers II/III (48.2 ± 3.8%, Fig. 1D and V (39.1 ± 3.7%, Supplementary Fig. S2A; Supplementary Table 2). The percentage of synpo-positive AIS stabilized from P35 in layer II/III (30.5 ± 1.2%, Fig. 1D) and from P21 in layer V (33.1 ± 4.7%, Supplementary Fig. S2A; Supplementary Table 2) and stayed high in adult animals (33.4 ± 4.9% for layer II/III, Fig. 1D; 36.1 ± 3.9% for layer V, Supplementary Fig. S2A; Supplementary Table 2). Of note, the peak in the percentage of synpo-positive AIS around P15 is preceded by the eye-opening of mice occurring between P13–14 (C57BL/6JR) strain; Gutzmann et al. (2014)). This relationship suggested a role for visual input in the developmental expression of synpo in V1.

The Number, but not the Average Size of Synpo Clusters per AIS Increases during Development
Next, we studied the number (Fig. 2A, C) and the size (Fig. 2B, D) of synpo clusters per AIS during visual cortex development. The time course for the number of synpo clusters per AIS was highly similar to the time course observed for the percentage of
synpo-positive AIS in V1 (c.f. Fig. 1D): the lowest number of synpo clusters per AIS were counted at P10 (1.5 ± 0.04 for layer II/III, Supplementary Table 2) and highest numbers between P15 (3.1 ± 0.2 for layer V, Supplementary Fig. S2B, Supplementary Table 2) and P21 (3.3 ± 0.3 for layer II/III, Fig 2A; Supplementary Table 2). This was observed in layers II/III (Fig. 2A) as well as in layer V (see Supplementary Fig. S2B).

Since synpo-positive COs can vary in length (Bas Orth et al. 2007; Sanchez-Ponce et al. 2011), we quantified the size of synpo clusters within individual AIS (Fig. 2B, D). Surprisingly, the average cluster size was constant over the entire time period (between 0.4 ± 0.01 μm² and 0.5 ± 0.02 μm² for layer II/III, between 0.4 ± 0.01 μm² and 0.5 ± 0.05 μm² for layer V; Supplementary Table 2). Correlation analysis of synpo cluster numbers and synpo cluster size per AIS (6 animals per age group) revealed a weak negative correlation between size and number of synpo clusters per AIS ($r = -0.3361, P < 0.0001$; Supplementary Table 3). In comparison, synpo cluster numbers did not show any differences in the nonsensory cingulate cortex control whereas synpo cluster sizes significantly increased only from P10 to P28 (P10 vs. P28: 0.7 ± 0.03 μm² vs. 0.9 ± 0.05; Supplementary Fig. S7).

We conclude from these data that the increase in the percentage of synpo-positive AIS goes hand-in-hand with an increase in the average number of clusters per AIS. AIS with larger synpo clusters have a slightly lower average number of synpo clusters per AIS. The average size of all clusters, however, stays unchanged over time. Similar findings were obtained for layer V (see Supplementary Fig. S2C). The development of synpo expression coincides with sensory input and is not observed in nonsensory cortex.

Visual Deprivation during Cortical Development Changes the Size but not the Number of Synpo Clusters in the AIS

Because of the correlation of the percentage of synpo-positive AIS as well as the average number of synpo-positive clusters/ AIS with the time of eye-opening, we hypothesized that visual input influences synpo cluster development during the CP. To address this question, we compared synpo cluster changes in the AIS of V1 neurons in control animals held under normal light/dark cycle conditions with those of animals reared under visual deprivation conditions for 21, 28, and 35 days. To...
additionally test whether dark deprivation has an effect on synpo cluster development after CP closure, we visually deprived P60 animals for 1 week until P67 (P > 55 (dark); Fig. 3). With regard to the number of clusters per AIS, dark rearing did not have any effect on layer II/III neurons during the CP (e.g., for P28 control vs. dark: 3.2 ± 0.1 vs. 3.2 ± 0.1; Fig. 3A, C). In adult visually deprived animals, a significant decrease in the number of synpo clusters was observed when compared to controls (P > 55 control vs. dark: 3.0 ± 0.1 vs. 2.1 ± 0.1; Fig. 3A). In layer V, a slight increase in cluster numbers was observed during the CP in P21 animals (P21 control vs. dark: 2.5 ± 0.1 vs. 3.0 ± 0.1, Supplementary Fig. S3A; Supplementary Table 2). Interestingly, with regard to the size of synpo clusters per AIS, dark rearing had a robust effect: synpo cluster size was increased in all...
Figure 3. Changes of synpo cluster size, but not number in AIS after sensory deprivation. (A) Visually deprived mice from P0 to 21, P0 to 28, and P0 to 35, as well as the P0–28 reversal group (dark rearing until P28, 1 week light exposure until P35) did not exhibit significant changes in number of synpo clusters in AIS of layer II/III when compared with P21, P28, and P35 control animals, respectively. Adult animals placed in darkness from P60 to 67 showed significant reduction in synpo cluster number, which incidentally goes in hand with a significant increase in synpo cluster size (compare to Fig. 3B). (B) Visual deprivation for 3, 4, and 5 weeks (P0–21, P0–28, and P0–35) as well as deprivation for one week from P60–67 resulted in significant increases in synpo cluster size in AIS of layer II/III. This effect was not reversible after light exposure for 1 week (P0–28, dark + rever). For A + B = Vertical bars indicate minimum and maximum data points. Dots delineate individual data points. Horizontal bars indicate the median. n = 6 animals, a minimum of 100 AIS per age and animal. Wilcoxon rank-sum test and Kruskal–Wallis 1-way analysis with Dunn’s post hoc test. *P ≤ 0.05 (C) Representative immunostaining for no change in cluster number with synpo (green), βIV-spectrin (red), and NeuN (blue) in layer II/III neurons of P28 control mice. Panel 1 is a maximum intensity projection of a confocal stack showing 2 neurons (asterisks highlight somata) with AIS containing 3 and 2 synpo clusters, respectively (arrows). Panel 2 (0° view) and 3 (25° tilt of same AIS) result from image reconstruction with Imaris. Both panels show higher magnification of the boxed region in panel 1 after deconvolution and volume reconstruction. Scale bar C1 = 10 μm, C2 + 3 = 2 μm. (D) Representative immunostaining for cluster size increase in layer II/III neurons of dark-reared P28 mice with synpo (green), βIV-spectrin (red), and NeuN (blue). Panel 1 is a maximum intensity projection of a confocal stack showing neurons (asterisk highlights soma, arrows along AIS indicate single, smaller synpo clusters) with AIS containing larger synpo clusters (outlined by arrowheads). Panel 2 (25° view) and 3 (180° tilt of same AIS) show higher magnification of the boxed region in panel 1 after deconvolution and volume reconstruction. Open arrowheads outline synpo clusters outside of the AIS, which only become visible in 3D rendering. White arrowheads outline larger synpo clusters inside the AIS. Arrows indicate single, smaller synpo clusters. Scale bar D1 = 10 μm, D2+3 = 2 μm.
groups during the CP investigated in both layers II/III and layer V (e.g., for P28 control vs. dark: 0.4 ± 0.02 μm² vs. 0.5 ± 0.02 μm² for layer II/III, 0.4 ± 0.03 μm² vs. 0.5 ± 0.02 μm² for layer V, Fig. 3B, D, see Supplementary Fig. S3B, Supplementary Table 2). Synpo clusters also increased in size in layer II/III AIS of visually deprived adult animals (P > 55 control vs. dark: 0.4 ± 0.03 μm² vs. 0.7 ± 0.03 μm²; Fig. 3B). No similar effect was observed in the nonsensory cingulate cortex control. Here, dark rearing did not have an effect on synpo cluster numbers or sizes (for P28 control vs. dark: 2.7 ± 0.1 vs. 2.9 ± 0.2 and 0.9 ± 0.1 μm² vs. 0.9 ± 0.1 μm²; see Supplementary Fig. S7).

After observing these changes, we attempted to reverse the visual deprivation-induced changes and exposed animals reared in the dark for 28 days to normal light conditions for 1 week (i.e., total observation time 35 days). We compared this “reversal” group with P35 control animals as well as P35 dark-reared animals. We speculated that an exposure of the dark-reared animals to light might shorten the average cluster size/AIS again. This was not the case, since the effect of dark rearing on synpo cluster size/AIS could not be reversed using this protocol. Rather, synpo cluster sizes were comparable to synpo cluster sizes found in animals reared for 35 days under visual deprivation conditions. (0.6 ± 0.03 μm² (P28 reversal) vs. 0.6 ± 0.03 μm² (P35 dark) for layer II/III, 0.7 ± 0.02 μm² (P28 reversal) vs. 0.7 ± 0.02 μm² (P35 dark) for layer V, Fig. 3B, Supplementary Fig. S3B).

The Presence of Synpo Clusters within AIS Correlates with Changes in AIS Length During Development

Since the presence of synpo in the AIS likely affects its function, we hypothesized that the presence of synpo in an AIS during the developmental period may also affect its length (Gutzmann et al. 2014). This appeared to be a plausible assumption because AIS length is tightly correlated with neuronal activity and thus a good structural correlate of neuronal function (Kuba et al. 2010; Evans et al. 2015; Gullede and Bravo 2016). Accordingly, we analyzed changes in AIS length in the subset of synpo-positive AIS in layers II/III (Fig. 4A, C) and in layer V of V1 (see Supplementary Fig. S4A). We found that synpo-positive AIS undergo a different morphological maturation than previously described for the entire AIS population in V1 (c.f. Fig. 5A; data from Gutzmann et al. (2014)). At P10, synpo-positive AIS showed a length of 29.8 ± 0.7 μm and 26.3 ± 0.5 μm in layers II/III and V, respectively (Fig. 4A, see Supplementary Fig. S4A, Supplementary Table 2). These values decreased and reached a plateau after P15/P21 (P21: 22.7 ± 1 μm for layer II/III, Fig. 4A; 19.7 ± 0.8 μm for layer V, Fig. S4A; Supplementary Table 2). In direct comparison with the tri-phasic length maturation of the entire AIS population (c.f. Fig 5A), the subset of synpo-expressing AIS lacks phase 1 (length increase until P10/15) and phase 2 (length decrease at P28). The average length of synpo-expressing AIS was also shorter compared to the entire AIS population in layer II/III and V (37.6 ± 1.6 μm, layers II/III for P15-10; data from Gutzmann et al. (2014)). Compared to the total population of AIS (c.f. Fig. 5A; data from Gutzmann et al. (2014)), these findings suggest that the presence of synpo clusters within AIS reduces and stabilizes AIS length.

Synpo Cluster-Containing AIS Elongate in Response to Visual Deprivation

Next, we examined whether the length of AIS with synpo clusters depends on visual input during the CP. Using a visual deprivation protocol, mice were reared in complete darkness from birth until P21, P28, and P35, respectively, and were visually deprived during adulthood from P60-67 (P > 55 (dark)). Under all dark rearing conditions during the CP, a significant increase in the length of synpo-positive AIS was seen in both layers II/III and V compared with controls (e.g., for P28 control vs. dark: 22.8 ± 0.2 μm vs. 27.8 ± 0.9 μm for layer II/III, 19.2 ± 1.3 μm vs. 23.2 ± 1.2 μm for layer V, Fig. 4B, D, Fig. S4B, Supplementary Table 2). In contrast, visual deprivation for one week after CP closure did not lead to an AIS length increase in adult animals (P > 55 control vs. dark: 22.3 ± 1.3 μm vs. 24.1 ± 0.7 μm; Fig. 4B).

Along with AIS lengthening, an increase in relative βIV-spectrin concentration was observed in AIS of visually deprived animals (see Supplementary Fig. S6). In the nonsensory cortex, dark rearing did not result in an increase of AIS length (for P28 control vs. dark: 27.8 ± 0.9 μm vs. 26.1 ± 1.3 μm; see Supplementary Fig. S7).

Thus, visual deprivation increases the length of synpo-expressing AIS in V1 during postnatal development within the time frame of the CP, in line with earlier reports demonstrating an inverse relationship between neuronal activity and AIS length (Kuba et al. 2010; Gullede and Bravo 2016). Size frequency histograms highlighted that visual deprivation before CP closure led to an AIS length distribution similar to that in young animals and thus appeared to prevent AIS structural maturation (see Supplementary Fig. S4C–K).

Finally, we studied mice treated according to the reversal protocol (P0–28 dark-reared, 1 week of light; i.e., 35 days total). We compared the AIS length of these mice to the AIS length of mice reared for 35 days under control or dark conditions. Similar to what we had seen for synpo cluster size changes/AIS, visual deprivation-induced AIS length changes were not reversible by this protocol and the AIS length of these mice stayed at the level of P0–35 dark-reared mice (29.6 ± 0.6 μm (P28 reversal) versus 28.6 ± 0.5 μm (P35 dark) for layer II/III, 26.5 ± 0.8 μm (P28 reversal) vs. 24.0 ± 0.5 μm (P35 dark) for layer V, Fig. 4B, Supplementary Fig. S4B).

AIS Length Maturation is Impaired in Synpo KO Mice during Development and After Sensory Deprivation

Since our findings indicate an AIS length-stabilizing effect of synpo (see above), we hypothesized that this should be different in the absence of synpo. Accordingly, we performed loss-of-function experiments using synpo KO mice lacking synpo and consequently, the CO (Bas Orth et al. 2007). The same time points and conditions (light/darkness) as for the wildtype mice were used to study AIS length changes. Similar to wildtype animals (Gutzmann et al. 2014); (Fig. 5A, see Supplementary Fig. S5A), the developmental time course analysis revealed that the AIS length of synpo KO mice increased during phase 1 from P1 (19.8 ± 0.7 μm) to P10/15 and (P15: 38.0 ± 1.0 μm) in layer II/III (Fig. 5B, Supplementary Table 2) and V (P1: 19.7 ± 1.2 μm, P10: 30.8 ± 1.4 μm, Supplementary Fig. S5B; Supplementary Table 2). After the time of eye-opening, however, the 2 curves significantly differed. Whereas wildtypes showed a rapid decrease of AIS length (phase 2: from 37.7 ± 1.3 μm (P15) to 21.9 ± 0.6 μm (P28) in e.g., layer II/III; data from (Gutzmann et al. 2014); Fig. 5A, Supplementary Fig. S5A), this was significantly different in synpo KO mice (from 38.0 ± 1.0 μm (P15) to 31.9 ± 1.6 μm (P28) in for example, layer II/III Fig. 5B, Supplementary Fig. S5B). At adult stages (Phase 3; Gutzmann et al. (2014); Fig. 5A, Supplementary Fig. S5A; Fig. 5B, Supplementary Fig. S5B), both groups stabilized around a similar AIS length (e.g., ~25–30 μm). Thus, synpo KO mice showed a less dynamic maturation of
Figure 4. Maturation and elongation of synpo-expressing AIS during visual cortex development and after sensory deprivation. (A) AIS expressing synpo did not undergo dynamic length maturation in layer II/III during development. Length of synpo-expressing AIS increased until a maximum at P10, shortly before eye-opening between P13–14, and continuously decreased during further postnatal development, showing significant changes only at P35 and P > 55. (B) Mice were visually deprived from P0 to 21, P0 to 28, and P0 to 35 as well as during adulthood from P60 to 67. All conditions during the CP (P21, P28, and P35) resulted in a significant length increase of synpo-expressing AIS in layer II/III as compared to P21, P28, and P35 controls, respectively. No changes in AIS length were observed for the adult deprivation group (P60–67). Vertical bars indicate minimum and maximum data points. Dots delineate individual data points. Horizontal bars indicate the median. n = 6 animals, a minimum of 100 AIS per age and animal. Wilcoxon rank-sum test and Kruskal–Wallis 1-way analysis with Dunn’s post hoc test *P ≤ 0.05. (C) Representative immunostaining of layer II/III neurons of P28 control mice using antibodies against synpo (green), βIV-spectrin (red), and NeuN (blue). Panel 1 is a maximum intensity projection of a confocal stack of a neuron (asterisk highlights soma) with an AIS containing 2 synpo clusters (arrows). Panels 2 (0° view) and 3 (30° tilt of same AIS) result from image reconstruction with Imaris. Both panels show higher magnifications of the neuron illustrated in panel 1 after deconvolution and volume reconstruction. Scale bar C1 = 5 μm, C2 + 3 = 3 μm. (D) Representative immunostaining of longer AIS in layer II/III neurons of dark-reared P28 mice with synpo (green), βIV-spectrin (red), and NeuN (blue). Panel 1 is a maximum intensity projection of a confocal stack showing a neuron (asterisk highlights soma) with an AIS with one very large synpo cluster (outlined by arrowheads) and other smaller clusters (arrows). Panels 2 (0° view) and 3 (30° tilt of same AIS) show higher magnifications of the neuron illustrated in panel 1 after deconvolution and volume reconstruction. Scale bar D1 = 6 μm, D2 + 3 = 4 μm.
their AIS compared with that of the entire AIS population in wildtype mice.

Finally, we studied the effect of dark rearing on synpo KO mice using the same time points as for wildtype mice, i.e., 3 weeks (P0–21), 4 weeks (P0–28) and 5 weeks (P0–35). Whereas visual deprivation had caused an increase in the length of synpo-positive AIS in wildtype mice (Fig. 4B, see Supplementary Fig. S4B), the opposite effect was seen in synpo-deficient AIS of synpo KO mice (e.g., for P28 control vs. dark: 31.9 ± 1.0 μm vs. 23.9 ± 1.3, micrometre for layer II/III, 26.4 ± 1.0 μm vs. 22.4 ± 0.8 μm for layer V, Fig. 5C, Supplementary Fig. S5C). The length of AIS in layers II/III significantly decreased in the first 2 groups (P21 and P28, Fig. 5C–E, Supplementary Table 2) and stayed constant in the 5 weeks group after visual deprivation. AIS length in layer V changed in P28 and P35 dark-reared animals (see Supplementary Fig. S5C, Supplementary Table 2). The decrease seen in the P0–28 group was not reversible following light exposure (25.4 ± 0.3 μm (P28 reversal) versus 25.8 ± 2.2 μm (P35 dark) for layer II/III, 23.2 ± 0.4 μm (P28 reversal) vs. 22.4 ± 1.6 μm (P35 dark) for layer V Fig. 5C, Supplementary Fig. S5C).

Figure 5. AIS length changes in synpo knockout mice during visual cortex development and after sensory deprivation. (A) AIS of layer II/III neurons in the visual cortex of wildtype mice underwent a tri-phasic length maturation during development. AIS were elongated during eye-opening at P10/15, shortened at P28 and re-elongated at P35 (original data modified from Gutzmann et al., 2014, with permission). (B) AIS in synpo KO mice lacked the distinct shortening at P28 and did not exhibit tri-phasic AIS length maturation. AIS were shortest at P1 and underwent length increase during further postnatal development until a maximum from P10 to P15 during eye-opening. Afterwards, a continuous and significant AIS length shortening occurred and AIS length stabilized during adulthood. (C) Mice were dark-reared until P21, P28, and P35. Visual deprivation led to a decrease in length of P21 and P28 animals. This decrease in AIS length could not be reversed. Vertical bars indicate minimum and maximum data points. Dots delineate individual data points. Horizontal bars indicate the median. n = 6 animals, a minimum of 100 AIS per age and animal. Wilcoxon rank-sum test and Kruskal–Wallis 1-way analysis with Dunn’s post hoc test *P ≤ 0.05. (D) and (E) AIS of layer II/III synpo KO mice of P28 control condition (D) and after sensory deprivation until P28 (E); sections immunolabeled against βIV-spectrin (green), NeuN (red), and synpo (blue). AIS shortening was observed in dark-reared synpo KO mice. Scale bar for D and E (shown in E) = 20 μm.
Discussion

The AIS is a functionally important and highly plastic axonal microdomain. Since the length of the AIS is tightly linked to neuronal activity (e.g., Yoshimura and Rasband 2014; Yamada and Kuba 2016), changes in AIS length are structural indicators of changes in neuronal function. In work leading to this study, we have shown that changes in AIS length correlate with the maturation of neurons in the visual cortex (V1) during development and with eye-opening (Gutzmann et al. 2014). These data were first evidence for structural AIS plasticity during the critical period of cortical plasticity (CP) in V1 development.

In the present study, we extended our earlier investigations and asked whether the molecular and cellular composition of the AIS also plays a role during this period of visual cortex plasticity. We focused on synpo, an actin-regulatory molecule essential for the formation of the CO within the AIS (Bas Orth et al. 2007). Synpo and the CO are good candidates for influencing AIS structural plasticity since they could have effects on the AIS cytoskeleton and/or local Ca\(^{2+}\)-trafficking (Benedeczky et al. 1994; Bas Orth et al. 2007). Our findings on the role of synpo during V1 development can be summarized as follows: (1) Synpo-positive AIS are first seen by P10. The percentage of synpo-positive AIS reaches a maximum (~50%) around the time of eye-opening and decreases thereafter to adult levels (~35–40%). (2) With maturation, synpo-positive AIS show an increase in cluster number, but not in size. (3) In contrast to the population of all AIS, the subpopulation of synpo-positive AIS does not show changes in AIS length during V1 development. (4) Dark rearing during the critical period results in an increase in synpo cluster size, but not number as well as in elongated AIS. Visual deprivation in adulthood increases synpo cluster size along with a decrease in synpo cluster numbers. AIS length remains unchanged (5) The effects of dark rearing are not reversible by light for 1 week after P28. (6) Synpo KO mice fail to show the decrease in AIS length following eye-opening and, furthermore, show a decrease in AIS length under conditions of dark rearing.

Taken together, our data suggest that the presence of synpo stabilizes AIS in the visual cortex (Fig. 6): before eye-opening, synpo cluster numbers increase with neuronal maturation. Following eye-opening, AIS with synpo maintain their length. Under deprivation conditions, synpo-positive AIS elongate before CP closure. In contrast, deprivation conditions shorten AIS lacking synpo. The presence of synpo may thus provide stability to the AIS of neurons during periods of functional perturbations.

The Presence of Synpo in the AIS of V1 Neurons is a Sign of Structural Maturation

Synpo is postnatally expressed in rodent brain (Mundel et al. 1997; Czarnecki et al. 2005). The developmental time course of synpo expression was studied previously in mouse hippocampus using in situ hybridization and immunolabeling (Czarnecki et al. 2005). This revealed a tight correlation between synpo mRNA expression and neuronal differentiation. Synpo immunolabeling dramatically increased during the period of spine formation and stabilization and was most prominently expressed by mature neurons and in large spines, in line with its role in adult synaptic plasticity (Deller et al. 2003; Vlachos et al. 2013). Of note, in this earlier study, the localization of synpo in the AIS was not investigated, since this subcellular localization of synpo was unknown and first described 2 years later (Bas Orth et al. 2007).

In our study on V1, the first synpo clusters were seen during the first postnatal week, most likely in spines. In the AIS, synpo appeared slightly later (by P10) and increased with neuronal maturation. Of note, maturation increased the percentage of neurons expressing synpo in their AIS and the number of synpo clusters per AIS, but not the size of individual synpo clusters. In parallel with this expression of synpo, we saw a shift in the cumulative length distribution of synpo-positive AIS. They shifted from a heterogeneous length distribution characteristic for immature AIS (Gutzmann et al. 2014) to a homogenous length distribution at P15, which is characteristic for mature AIS in adult animals (Gutzmann et al. 2014). This suggests that synpo expression in the AIS is a sign of structural maturity, similar to what has been described for synpo expression in the spine microdomain (Mundel et al. 1997; Czarnecki et al. 2005).

Synpo-Positive AIS are more Stable during Visual Cortex Development

An important structural and functional parameter of the AIS is its length. As has been shown using functional and computational approaches (reviewed in Adachi et al. 2015), the excitability and the length of AIS are tightly linked: elongated AIS have larger Na\(^+\) and K\(^+\) conductances and a higher membrane capacitance, resulting in higher excitability. Interestingly, the activity of neurons and their excitability (and length) of AIS are inversely correlated to neuronal activity. Higher neuronal activity results in shorter AIS whereas lower activity is linked to AIS elongation (Kuba et al. 2010; Yamada and Kuba 2016). This relationship suggests that AIS length changes subserve a homeostatic purpose and contribute to the homeostatic regulation of neuronal activity of neurons (Adachi et al. 2015).

In our previous work on AIS in developing V1 (Gutzmann et al. 2014), we studied changes in AIS length of all neurons. As the length of AIS is an important structural parameter, we demonstrated that AIS in V1 undergo 3 consecutive phases of structural maturation, which correlate with sensory activity during development: AIS were longest during eye-opening (phase 1), shortened around the peak of the CP at P28 (phase 2), and re-elongated with CP closure at P35 (phase 3). Mature AIS showed constant length throughout adulthood (Gutzmann et al. 2014). In the present study, we have addressed the question how the length of the subpopulation of synpo-positive AIS changes during V1 development. We compared these data with those of Gutzmann et al. (2014) and were surprised to find that synpo-positive AIS were more stable and lacked the dynamic second phase we had observed previously. Together with the fact that synpo-positive AIS were shorter than the average AIS, our observations suggest that synpo-positive AIS are present in active neurons of V1, which may have integrated into the developing visual cortex network (Hensch 2005). Synpo could either be a maturation marker of those neurons or could be the means by which integrated and regularly active mature neurons stabilize their AIS (see below).

Synpo and the CO of the AIS may Regulate Calcium Trafficking in the AIS during V1 Development

The cellular and molecular mechanisms linking synpo to AIS function and/or plasticity are unknown, but likely linked to the Ca\(^{2+}\) storage and sequestering function of the CO. This specialized axonal organelle, which is characterized by stacked ER bound together by an unknown electron dense substance (Benedeczky et al. 1994), requires synpo for its formation (Bas Orth et al. 2007). The neuronal ER network acts as a Ca\(^{2+}\) reservoir.
modulating neuronal Ca\textsuperscript{2+} signals (Simpson et al. 1995) and has been suggested to play an active role in neuronal Ca\textsuperscript{2+}-signaling through activating Ca\textsuperscript{2+}-sensitive channels (Berridge 1998). The sarco/ER Ca\textsuperscript{2+} ATPase (SERCA) type 1 Ca\textsuperscript{2+} pump, the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor Ca\textsuperscript{2+} channel (IP\textsubscript{3}R), and the ryanodine receptor (RyR) are associated with synpo and/or the CO (Benedeczky et al. 1994; Sanchez-Ponce et al. 2011; King et al. 2014; Anton-Fernandez et al. 2015). These channels are potentially the first to be activated by Ca\textsuperscript{2+} that enters the AIS during AP firing (Bender and Trussell 2012). Intracellular Ca\textsuperscript{2+} storage loading through, for example, SERCA increases RyRs and IP\textsubscript{3}Rs sensitivity and consequently, might amplify local cytosolic Ca\textsuperscript{2+} transients through Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (Berridge 1998), which in turn could impact on neuronal activity (reviewed in Hille 1986; Berridge (1998)) and, thus, AIS length.

Changes in Synpo Expression Correlate with Eye-Opening and may be Linked to Experience-Dependent Modifications during the Critical Period

During murine V1 development, synpo is highest expressed at P15, right after eye-opening. This correlation suggests a role for synpo or the CO in the reorganization of cortical connections during the CP. How could synpo or the CO be mechanistically involved in such experience-driven modifications? A number of cellular and molecular mechanisms have been identified that underlie network modifications during the CP of the visual cortex (Hensch 2005). One of the most important signaling molecules appears to be Ca\textsuperscript{2+}: In cat visual cortex, developmental changes in the laminar distribution of extracellular Ca\textsuperscript{2+} suggested that experience-dependent modifications are triggered by Ca\textsuperscript{2+} influx (Bode-Greuel and Singer 1991). In rat visual cortex, a developmental switch from low-voltage-activated Ca\textsuperscript{2+} currents to high-voltage-activated Ca\textsuperscript{2+} currents appears around P12 (Tarasenko et al. 1998). Such high-voltage-activated Ca\textsuperscript{2+} currents are responsible for AP hyperpolarization (Umemiya and Berger 1994). As the CO is a putative regulator of local AIS Ca\textsuperscript{2+}-trafficking, its presence within an AIS could influence axonal membrane potential properties and neuronal excitability. Furthermore, synpo and Ca\textsuperscript{2+}-signaling could modulate GABA\textsubscript{A} receptors at the AIS (Llano et al. 1991; Benedeczky et al. 1994; King et al. 2014). GABA\textsubscript{A} receptors are the postsynaptic target of Chandelier terminals that specifically form axo-axonic synapses at the AIS (Woodruff et al. 2010). Several studies have shown the importance of GABAergic transmission for proper cortical maturation during CPs (Hensch 2005). Thus, lack of synpo might lead to functional changes of the excitation/inhibition balance in cortical networks during development. In sum, synpo and the CO may not only be markers for mature neurons, but may play a functional role for cortical rewiring after eye-opening.
Sensory Deprivation causes Remodeling of Synpo-Positive Structures in the AIS

Sensory deprivation during the early developmental period results in longer AIS compared with age-matched controls (reviewed in Adachi et al. (2015)). This also holds true for V1, where AIS of animals reared under visual deprivation conditions were found to be ~150% longer than AIS of animals reared under control conditions (Guttmann et al. 2014). In the present study, we focused on the subpopulation of synpo-positive AIS and obtained similar results: synpo-positive AIS of dark-reared mice were ~125% longer than AIS of controls. Of note, the length of AIS from dark-reared animals corresponded to that of animals before eye-opening, suggesting that dark rearing does not elongate the AIS, but rather that increased sensory input and network activity shortens the AIS after eye-opening. Because the sensory stimulus is missing in dark-reared animals, the AIS of these mice stay abnormally long.

Interestingly, however, synpo-positive AIS showed changes in synpo clusters under conditions of sensory deprivation: both, dark rearing before CP closure and visual deprivation in adulthood resulted in an increase in the size, but number of synpo clusters was changed only in some deprivation conditions showing either increase (during CP; P21 in layer V) or decrease (in adulthood; P > 55 in layer II/III) when compared with age-matched controls. This was a particularly striking result, since under control conditions the average size of synpo clusters was constant over the entire investigated time period. As this increase in size was mirrored by a reduction in AIS cluster numbers in adulthood, this finding suggests an increase in the total amount of synpo in these AIS, possibly due to fusion of existing synpo clusters. What could be the consequence of such a change? It is well-known that the ER and its associated structures are constantly undergoing remodeling (Schwarz and Blower 2016). Specifically, dendritic ER is subject to reversible fragmentation and expansion triggered by for example, Ca\(^{2+}\)-dependent mechanisms (Kucharz et al. 2013). ER expansion most likely provides increased Ca\(^{2+}\) storage capacity (Sammels et al. 2010). Likewise, our observed increase of synpo cluster size after visual deprivation probably serves to enhance the AIS-associated Ca\(^{2+}\) storage capacity. We speculate that this change could be functionally linked to the homeostatic response of the AIS seen under deprivation conditions (e.g., Yoshimura and Rasband (2014); Adachi et al. (2015)). Together with the long AIS, which increases the excitability of a neuron, an elongated CO could increase AIS Ca\(^{2+}\) currents. Both changes may be interpreted as homeostatic responses of maturing neurons in dark-reared animals aiming at counterbalancing the sensory deprivation-induced reduction in network activity. In adult animals, where AIS length remains constant even after deprivation, the mechanism might serve to alter the intrinsic Ca\(^{2+}\) storage capacity significantly, since homeostatic changes in excitability are no longer possible due to the now established, mature AIS length.

AIS of Synpo-Deficient Animals Show a Paradoxical Response to Dark Rearing

Finally, we addressed the question how the lack of synpo affects AIS length of V1 neurons during development and under sensory deprivation conditions. Similar to controls (Guttmann et al. 2014), synpo-deficient mice showed an initial maturation-dependent increase in AIS length until the time of eye-opening. With sensory input and increased network activity, shorter AIS were observed. Compared with controls (Guttmann et al. 2014), synpo-deficient mice lacked the strong decrease in AIS length around P28. More striking, however, was the response of synpo−/− AIS to visual deprivation. In dark-reared animals, a decrease in AIS length was seen at P21, P28, and P35. This was surprising in light of the fact that control animals showed an increase in AIS length under these conditions.

The differences in AIS dimensions between the synpo-mutants and the controls indicate differences in their network activity. Indeed, in an earlier study on the hippocampus of synaptodyn-deficient mice (Jedlicka et al. 2009), network activity was found to be increased in the mutants. An increased basal network activity could at least contribute to the decreased AIS length seen in the mutants. Electrophysiological recordings of network and single unit activity during the critical period of visual cortex development will be needed to verify this hypothesis. What is the cellular basis for the increased network activity seen in synpo-deficient mice? Data from the hippocampus suggest that lack of the CO in the AIS could play an important role and alter GABA\(_A\) receptor-mediated recurrent inhibition and GABAergic neurotransmission at the AIS (Jedlicka et al. 2009). The contribution of the spine apparatus organelle, which is also missing in synpo-deficient mice, is more complex. Basal synaptic transmission of synaptodyn−/− mice is normal and a synaptic-phenotype is seen only under plasticity conditions (Deller et al. 2003; Jedlicka et al. 2009; Vlachos et al. 2013; Zhang et al. 2013; Verbich et al. 2016). Thus, the absence of the spine apparatus may not play a major role for network activity under conditions of sensory deprivation, but may become relevant under conditions of sensory input and plasticity, that is, during the CP. Our data on the AIS of synpo-deficient mice indicate that absence of synaptopodin from visual cortical neurons affects plastic changes of AIS dimensions during the CP. The absence of the CO and—depending on the synaptic input to these neurons—absence of the spine apparatus are likely to contribute to this phenomenon at the cellular level.

Several studies have also shown that the integrity of AIS structural proteins anK and ßIV-spectrin as well as of voltage-gated channels can be regulated through extracellular Ca\(^{2+}\)-signaling mediated by the Ca\(^{2+}\)-dependent cysteine protease calpain (Schafer et al. 2009; Del Puerto et al. 2015; Benned-Jensen et al. 2016). This integrity had additional effects on the firing behavior of neurons (Benned-Jensen et al. 2016), indicating a further role of Ca\(^{2+}\)-signaling in modulating neuronal activity. If AIS structural integrity relies on extracellular Ca\(^{2+}\) influx into the AIS, the CO might also have direct impact on the modulation of AIS structural plasticity through the regulation of Ca\(^{2+}\) homeostasis and maintaining Ca\(^{2+}\) buffer capacity in a physiological range. An impairment in AIS integrity might thus be an additional reason for the observed paradoxical decrease of AIS length in response to dark rearing in mice lacking synpo and the CO.

Conclusions and Outlook

Our data suggest an important role for synpo and the CO during the critical phase of visual cortex development: Synpo is expressed in ~50% of AIS, developmental changes in synpo correlate with maturation and eye-opening, and synpo plasticity appears to be involved in homeostatic responses of sensory input-deprived V1 neurons to reduced network activity. Whether synpo is essential for the development of normal vision is unclear, however, since neither functional nor behavioral data on the visual system of synpo-deficient mice have

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yet been published. Based on our data on synpo in the developing V1 such investigations now appear to be warranted.

**Supplementary Material**

Supplementary data are available at *Cerebral Cortex* online.

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