1. Introduction

The ubiquitous second messenger molecule cyclic AMP (cAMP) is an important member of many signaling cascades in the central nervous system. cAMP signaling has been shown to be crucial for neuronal development, dendritic and axonal morphogenesis, and synaptic plasticity, and it modulates a broad range of cognitive functions, including working and reference memory (Lee, 2015; Ricciarelli and Fedele, 2018). Alterations in upstream and downstream components of the cAMP pathway have also been shown to affect behaviors including sociability and communication (Burgdorf et al., 2007; Fischer and Hammerschmidt, 2011; Wang et al., 2008). Conversely, abnormal cAMP signaling has been implicated in a range of neurodevelopmental and psychiatric disorders, several of which affect cognitive functions (Garcia et al., 2016; Havekes et al., 2015; Kelley et al., 2008; Kelly et al., 2009; Nestler et al., 2002; Ricciarelli and Fedele, 2018).

cAMP signaling occurs via two main downstream pathways, one that is protein kinase A (PKA)-dependent and another that is PKA-independent (Bos, 2003). PKA-independent cAMP targets include EPAC (exchange protein directly activated by cAMP) proteins (Bos, 2003) and cyclic nucleotide-gated channels. While much attention has been dedicated to the role of the PKA-dependent pathway in plasticity and cognitive behavior, relatively little is known about the roles of the PKA-independent mechanisms in the brain. EPAC2, also known as cAMP-GEFII or RapGEF4, is a brain-enriched guanine-nucleotide exchange factor that regulates GTPase activity of the small GTPase Rap and Ras and is highly enriched at synapses. Activation of EPAC2 has been shown to induce dendritic spine shrinkage and increase spine motility, effects that are necessary for synaptic plasticity. These morphological effects are dysregulated by rare mutations of Epac2 associated with autism spectrum disorders. In addition, EPAC2 destabilizes synapses through the removal of synaptic GluA2/3-containing AMPA receptors. Previous work has shown that Epac2 knockout mice (Epac2<sup>−/−</sup>) display abnormal social interactions, as well as gross disorganization of the frontal cortex and abnormal spine motility in vivo. In this study we sought to further understand the cellular consequences of knocking out Epac2 on the development of neuronal and synaptic structure and organization of cortical neurons. Using primary cortical neurons generated from Epac2<sup>+/+</sup> or Epac2<sup>−/−</sup> mice, we confirm that EPAC2 is required for cAMP-dependent spine shrinkage. Neurons from Epac2<sup>−/−</sup> mice also displayed increased synaptic expression of GluA2/3-containing AMPA receptors, as well as of the adhesion protein N-cadherin. Intriguingly, analysis of excitatory and inhibitory synaptic proteins revealed that loss of EPAC2 resulted in altered expression of vesicular GABA transporter (VGAT) but not vesicular glutamate transporter 1 (VGluT1), indicating an altered ratio of excitatory and inhibitory synapses onto neurons. Finally, examination of cortical neurons located within the anterior cingulate cortex further revealed subtle deficits in the establishment of dendritic arborization in vivo. These data provide evidence that loss of EPAC2 enhances the stability of excitatory synapses and increases the number of inhibitory inputs.
factor (GEF) for the small GTPase Rap and is the major EPAC protein expressed throughout development and in the adult brain (Kawasaki et al., 1998; Ulucan et al., 2007; Woolfrey et al., 2009). EPAC2 contains two cAMP-binding domains and a Rap-GEF domain, in addition to other domains. Binding of cAMP to the cAMP-binding domain enhances the catalytic activity of the GEF domain toward Rap in both EPAC1 and EPAC2 (Bos, 2003; Woolfrey et al., 2009). Work from our group has also shown that EPAC2 is required for the establishment and maintenance of basal dendritic arborization through its interaction with the small GTPase Ras during development (Srivastava et al., 2012b). Activation of EPAC2 in neurons with a mature cellular morphology results in the shrinkage of dendritic spines and synapse destabilization through the removal of GluA2/3-containing AMPA receptors from synapses (Woolfrey et al., 2009). Moreover, EPAC2 is a critical mediator of dopamine D1 receptor-mediated spine remodeling (Woolfrey et al., 2009).

Interestingly, EPAC2 activation can also be regulated by the adhesion protein neuriligin 3 (NL3), a protein associated with autism spectrum disorders (ASDs) (Woolfrey et al., 2009). Critically, rare coding variants of Epac2 have also been associated with ASDs (Bachelli et al., 2003), and these variants alter the ability of EPAC2 to regulate synaptic structure and function (Woolfrey et al., 2009). Interestingly, Epac2 knockout mice (Epac2−/−) displayed abnormal organization of the anterior cingulate cortex (ACC), reduced spine dynamics in vivo (Srivastava et al., 2012a; Viggiano et al., 2015) and specific deficits in social and communicative behaviors (Srivastava et al., 2012a). These behavioral deficits are also mirrored in mice lacking both Epac1 and Epac2 (Yang et al., 2012; Zhou et al., 2016). While these data indicate a role for EPAC2 in both developing and adult brain, a comprehensive examination of this protein’s role in synaptic organization in vitro and in vivo has yet to be performed.

In this study, we have used primary cortical cultures generated from Epac2−/− mice and wild-type littermates (Srivastava et al., 2012a) to examine the ability of cells to respond to cAMP stimulation. Furthermore, we have examined the impact of EPAC2 loss on the organization of synapses on cortical neurons. Specifically, we have focused on the synaptic presence of AMPA receptors and adhesion proteins known to directly or indirectly be associated with EPAC2. We further investigate whether loss of EPAC2 altered the ratio of excitatory and inhibitory synapses on neurons. Finally, as we have previously shown that loss of Epac2 alters the dendritic organization and spine dynamics of layer 2/3 and layer 5 cortical neurons, respectively, located in pre-motor and somatosensory areas (Srivastava et al., 2012a; Srivastava et al., 2012b), we examined whether knockout Epac2 alters the dendritic and synaptic morphology of layer 5 neurons located in the ACC. The result of these investigations indicates loss of EPAC2 impacts the abundance of AMPA receptor subunits and specific adhesion proteins at synapses. Moreover, Epac2−/− neurons display an increase in the number of inhibitory inputs. Finally, layer 5 ACC neurons display subtle alterations in dendritic arborization in Epac2−/− mice. Taken together, these data indicate that EPAC2 is required for the normal establishment of synapses, and influences the ratio of excitatory and inhibitory inputs to cortical neurons.

2. Materials and methods

2.1. Reagents

CAMP analog 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-CPT) was purchased from Tocris Bioscience (R&D Systems). Sources of antibodies are as follows: rabbit anti-Epac2 polyclonal (Cell Signaling Technology), rabbit anti-jasplakinolide (Sigma), rabbit anti-NL3 polyclonal (Santa Cruz Biotechnology), rabbit anti-GluA2/3 polyclonal (Millipore), mouse anti-VGAT polyclonal (Millipore), mouse anti-VGlut1 monoclonal (Millipore), rabbit anti-PSD-95 polyclonal (Millipore), mouse anti-GluA2 monoclonal (University of California-Davis/National Institutes of Health Neurorob Facility), mouse anti-PSD-95 monoclonal clone K28/43 (University of California-Davis/National Institutes of Health Neurorob Facility), chicken anti-GFP polyclonal (Abcam) and mouse anti-bassoon monoclonal (Abcam).

Epac2−/− mice (C57BL/6) were generated by Professor Susumu Seino of Kobe University (Shibasaki et al., 2007); this line was maintained by crossing heterozygous (Epac2+/−) mice. In order to label a subset of layer 5 neurons with green fluorescent protein (GFP), wild-type (Epac2+/+) and knockout (Epac2−/−) littermate mice were crossed with the Tg(Thy1-GFP)-2Jrs/J transgenic line (Jackson Labs) as previously described (Srivastava et al., 2012a; Viggiano et al., 2015). Tg(Thy1-GFP)2Jrs/J express GFP in a subset of layer 5 neurons throughout the neocortex; labelled neurons are ideal for morphological studies. This resulted in the generation of Epac2+/+,GFP and Epac2−/−,GFP mice; these animals were back-crossed for 9 generation before they were used in subsequent experiments. For studies examining synaptic and dendritic morphology in vivo, 8-week-old male Epac2+/+,GFP and Epac2−/−,GFP mice were used. Mice were used in accordance with ACUC institutional and national guidelines under approved protocols. Generation of the HA-EPAC2 was described previously (Woolfrey et al., 2009).

2.2. Culturing of primary cortical neurons from wild-type and Epac2−/− mice

Dissociated cultures of primary cortical neurons were prepared from Epac2 wildtype (+/+) or knockout (−/−) animals; cultures were prepared side by side, and comparisons made only between cultures grown in parallel. Cortical neuronal cultures, consisting of mixed sexes, were prepared from P0 mouse pup in accordance with ACUC institutional and national guidelines under approved protocols and as described before (Srivastava et al., 2012a; Srivastava et al., 2011). Briefly, mouse pups were euthanized by decapitation, brains were quickly removed, and cortical tissue was isolated, digested, and dissociated. Cells were plated onto 18 mm glass coverslips (No 1.5; 0117580, Marienfeld-Superior GmbH & Co.), coated with poly-o-lysine (0.2 mg/ml, Sigma), at a density of 3 × 105 cells per well equal to 857/mm2. Neurons were cultured in feeding media: neurobasal medium (21103049) supplemented with 2% B27 (17504044), 0.5 mM glutamine (25030024) and 1% penicillin/streptomycin (15070063) (all reagents from Life Technologies). Neuron cultures were maintained in presence of 200 μg/mL D,L-Amino-phosphonovaralate (D,L-APV, ab120004, Abcam) beginning on DIV (days in vitro) 4 in order to maintain neuronal health for long-term culturing and to reduce cell death due to excessive Ca2+ cytotoxicity via over-active NMDA receptors (Srivastava et al., 2011). Half media changes were performed twice weekly until desired age (DIV 23–25). A subset of primary cortical neurons were transfected with eGFP at DIV 21 for 2 days using Lipofectamine 2000 (11668027, Life Technologies) (Srivastava et al., 2011). Briefly, 2–4 μg of plasmid DNA was mixed with Lipofectamine 2000 and incubated for 4–12 h, before being replaced with fresh feeding media. Two days after transfection, cells were used for pharmacological treatment or immunocytochemistry (ICC).

2.3. Immunocytochemistry (ICC)

Neurons were washed in PBS and then fixed in 4% formaldehyde/4% sucrose PBS for 10 min at room temperature followed by incubation in methanol pre-chilled to ~ −20 °C for 10 min at 4 °C. Fixed neurons were then permeabilized and blocked simultaneously (2% Normal Goat Serum, 5425S, New England Biolabs and 0.1% Triton X-100) before incubation in primary antibodies overnight and subsequent incubation with secondary antibodies the following day (Srivastava et al., 2011).

2.4. Quantitative analysis of spine morphologies and immunofluorescence

Confocal images of double-stained neurons were acquired with a
Zeal 2+/+, GFP

2.6. Preparation of cortical tissue sections

Intensity of each band and normalizing to β-actin, for protein loading, quantification of bands was performed by measuring the integrated gray value from five regions of interest plus two standard deviations (Glyn and McAllister, 2006). Cultures that were directly compared were stained simultaneously and imaged with the same acquisition parameters. For each condition, 10–16 neurons from at least 3 separate experiments were used. Experiments were conducted blind to condition and on sister cultures. In the green/magenta color scheme, co-localization is indicated by white overlap.

2.5. Western blotting and sample preparation

Whole cell lysates were prepared from DIV 25 neurons generated from wildtype or knockout mice. Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.1% SDS (weight/volume), 1% Triton X-100 (volume/volume), 1% deoxycholate (weight/volume), and inhibitors), before being sonicated with 10 short bursts. Sample buffer was added to all samples, which were then denatured for 5 min at 95 °C and stored at −80 °C until used further.

Whole cell lysates and crude synaptosome fractions were prepared from either hemispheres from the same animals. Briefly, cortical tissue from 8-week-old male mice was dissected after they were sacrificed and homogenized using 10 strokes of a Teflon-coated homogenizer, followed by sonication, in either RIPA buffer (whole cell lysate) or homogenization buffer (320 mM sucrose; 5 mM Na2HPO4; 1 mM EDTA pH 8; and 10 mM HEPES pH 7.4 + protease inhibitors) and subsequently passed through a 21 gauge needle 15 times. To generate P2 fractions, cell lysates were centrifuged to remove the nuclear fraction and large cell organelles (P1 fraction), yielding the extranuclear fraction (S1). The supernatant was subjected to further fractionation by an additional spin, yielding the a S2 (supernatant) and crude synaptosome (P2; pellet) fractions. The P2 fraction was resuspended in homogenization buffer. Sample buffer was added to all samples, which were then denatured for 5 min at 95 °C and stored at −80 °C until used further.

All samples were subsequently separated by SDS-PAGE and analyzed by Western Blotting with antibodies against EPAC2 and β-actin. Quantification of bands was performed by measuring the integrated intensity of each band and normalizing to β-actin, for protein loading, using ImageJ.

2.6. Preparation of cortical tissue sections

In order to examine dendritic and synaptic structures in cortical layer 5, 8-week-old Epac2+/+,GFP and Epac2−/−,GFP mice were anesthetized with a ketamine/xylazine mixture and perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. All experiments were carried out in accordance with ACUC institutional and national guidelines under approved protocols. Brains were removed, postfixed overnight in 4% paraformaldehyde/PBS, and cryoprotected in 30% sucrose/PBS. Brains were then embedded in 3% agarose and sectioned coronally at 300 μm with a vibratome. Sections were mounted onto a glass slide and covered with a No.1.5 glass coverslip with 2 #1 coverslips (~150 μm thickness) placed either side of the section to avoid damage to the tissue (Srivastava et al., 2012b).

2.7. 2 photon laser scanning microscopy (2PLSM) imaging of fixed brain sections and quantitative morphological analysis

Fixed brain sections were imaged on an Olympus BX51-WIF upright, fixed-stage microscope using a Zeiss LD LCI PA 25×/0.8.NA multi-immersion lens (440842-9870-000000), and a Coherent Chameleon Ultra2 tunable (680 nm to 1080 nm) laser system utilizing Ti:sapphire, attenuated by two ConOptics Pockels cell electro-optic modulators. The scanning system software used was LaserSharp (BioRad). GFP-expressing cells were excited at 950 nm, and Z-stacks (100–200 images) were acquired at 500 lines per second, at a resolution of 1024 × 1024 pixels: at digital zoom = 1 (for dendritic arbors), xy pixel = 271 0.45 μm with 1 μm Z steps; at digital zoom = 3.6 (for dendritic spines), xy pixel = 0.13 μm with 0.75 μm Z-steps. Kalman corrections (N = 6) were applied to images acquired at zoom of 3.6. The ACC was identified and only GFP-expressing layer 5 pyramidal neurons in the ACC were imaged. Only cells exhibiting intact healthy secondary and tertiary apical and basal dendrites were imaged and used for quantification. Following acquisition, images were projected as 2-D Z-projections using MetaMorph for analysis of dendritic spines. For each condition, 1–2 cells from 6 to 8 animals imaged. Two dendrites between 50 and 100 μm in length per cell were measured: only spines on tertiary apical or secondary basal dendrites were imaged to reduce variability. Dendritic spine density (number of spines per 10 μm) as well as spine area, were calculated using MetaMorph. To examine dendrite arborization, z-stacks were maintained in 3 dimensions during tracing of dendritic arbors using the Neuromantic program (http://www.reading.ac.uk/neuromantic/) (Myatt et al., 2012). Briefly, neurites were digitally traced and subsequently reconstructed in 3D using Neuromantic. SWC data files, encoding the 3D reconstruction of the dendritic arbors, were exported and analyzed using L-measure (Scorrioni et al., 2008).

2.8. Statistical analysis

All statistical analysis was performed in GraphPad. Differences in quantitative immunofluorescence, dendritic spine number were probed by one-way-ANOVA’s with Tukey correction for multiple comparisons. Error bars represent standard errors unless stated otherwise.

3. Results

3.1. Epac2−/− neurons exhibit abnormal dendritic spine morphology in response to 8-CPT stimulation

Previous studies have demonstrated a role for an EPAC2-dependent regulation of dendritic spine morphology in response to cAMP stimulation (Woolfrey et al., 2009). Thus, we were interested to see if primary cortical neurons (DIV 21–23) from wild-type and Epac2 knockout mice differed in their ability to respond to cAMP. Western blotting of cell lysates from primary cultures confirmed loss of EPAC2 in knockout cultures (Fig. 1A). Next, we compared the dendrite density and size of dendritic spines in neurons from wild-type and Epac2−/− mice and found no difference in density, but an increase in spine area in neurons from knockout mice (Fig. 1B–C). When we stimulated neurons from wild-type and Epac2−/− mice with 8-CPT to mimic a PKA-independent cAMP signaling mechanism. Analysis of spine morphology revealed that 8-CPT caused shrinkage of dendritic spines in neurons from wild-type cultures but not Epac2−/− cultures (Fig. 1D–F). 8-CPT treatment caused no differences in spine density in
either wild-type or knockout neurons. Finally, we tested whether ectopic expression of EPAC2 could reverse enlarged dendritic spine size. Thus, we compared dendritic spine density and morphology in Epac2−/− neurons expressing HA-EPAC2 or not (Fig. 1G). Exogenous EPAC2 did not alter spine linear density; however, Epac2−/− neurons expressing HA-EPAC2 had significantly smaller spines compared to Epac2−/− cells (Fig. 1G, H). Taken together, these data indicate that knockout of Epac2 results in an increase in the number of large spines and abolishes cAMP-dependent regulation of dendritic spine morphology.

3.2. Epac2−/− neurons have increased synaptic levels of GluA2/3

We have previously shown that EPAC2 interacts with other postsynaptic proteins, including PSD-95 and GluA2 (Woolfrey et al., 2009). Moreover, EPAC2 regulates the trafficking of GluA2/3-containing glutamate receptors and AMPA receptor-mediated transmission (Woolfrey et al., 2009). To examine whether loss of EPAC2 altered the synaptic content of AMPA receptors, we generated primary cultures of cortical neurons from wild-type and Epac2−/− mice and immunostained them for the synaptic protein PSD-95 and GluA2/3-containing AMPA receptors. When we examined PSD-95 puncta density, we found no differences between the genotypes (Fig. 2A–B), indicating that loss of Epac2 did not affect the density of synapses. This is consistent with the observation that Epac2−/− neurons do not have altered spine number. However, when we examined GluA2/3 puncta density, we found a significant increase in neurons from Epac2−/− mice compared to wild-type mice (Fig. 2A, C). Furthermore, GluA2/3 clusters were larger in Epac2−/− cultures (Fig. 2A, D). These effects on puncta size and density were also accompanied by an increase in the number of PSD-95 and GluA2/3-containing AMPA receptors. When we examined PSD-95 puncta density, we found no differences between the genotypes (Fig. 2A–B), indicating that loss of Epac2 did not affect the density of synapses. This is consistent with the observation that Epac2−/− neurons do not have altered spine number. However, when we examined GluA2/3 puncta density, we found a significant increase in neurons from Epac2−/− mice compared to wild-type mice (Fig. 2A, C). Furthermore, GluA2/3 clusters were larger in Epac2−/− cultures (Fig. 2A, D). These effects on puncta size and density were also accompanied by an increase in the number of PSD-95 and GluA2/3 colocalized puncta in Epac2−/− neurons (Fig. 2A, E), indicating an increased presence of GluA2/3 at synapses. These data further demonstrate that EPAC2 regulates the synaptic content of GluA2/3-containing AMPA receptors.

3.3. Epac2−/− neurons display altered adhesion protein expression at synapses

Loss of Epac2 either in vitro or in vivo results in the stabilization of synapses, which is accompanied by an increase in the number of large spines (Srivastava et al., 2012a; Woolfrey et al., 2009). Synapse stability is coordinated by adhesion molecules such as N-cadherin and the neuroligins (Jang et al., 2017). We have previously shown that NL3 forms a protein complex with EPAC2 at synapses (Woolfrey et al., 2009). Moreover, Rap1 regulates the presence of N-cadherin at synapses (Xie et al., 2008). Therefore, we reasoned that, as Epac2−/− cultures displayed abnormal dendritic spine morphologies and that EPAC2 is a direct regulator of Rap1, that neurons lacking this protein may also display altered expression of adhesion proteins at synapses. We first examined the presence of NL3 and the pre-synaptic and active zone marker bassoon in wild-type and knockout cultures. Assessment of the linear density of bassoon revealed no difference between genotypes (Fig. 3A–B). This is consistent with there being no alteration in synapse density in Epac2−/− neurons. Interestingly, no difference in NL3 puncta density or size was observed between wild-type and Epac2 knockout cultures (Fig. 3C). However, when we assessed cluster size, we found that both NL3 and bassoon puncta were larger in neurons from Epac2−/− cultures (Fig. 3D, E). This is in line with our observation that Epac2−/− neurons have larger dendritic spines.

N-cadherin is known to stabilize synapses and N-cadherin cluster size is directly proportional to dendritic spine size (Xie et al., 2008). We therefore examined whether loss of EPAC2 would impact the clustering of N-cadherin at synapses. Again, we observed no difference in PSD-95 linear density between genotype (Fig. 4A and B). Interestingly, we also observed no changes in the linear density of N-cadherin puncta between wild-type and knockout cultures (Fig. 4A and C). However, when we examined N-cadherin puncta in more detail, we found that the cluster size was significantly increased in Epac2−/− cultures (Fig. 4A and D). Critically, when we examined the density of colocalized PSD-95 and N-cadherin puncta, we found a significant increase in colocalized puncta in Epac2−/− neurons, indicating an enrichment of this adhesion protein at synapses (Fig. 4A and E). These data suggest that Epac2−/− neurons have an increased amount of N-cadherin at synapses, consistent with an apparent increase in synapse stabilization.

3.4. Epac2−/− neurons have an altered ratio of excitatory and inhibitory synaptic inputs

EPAC2 has been localized to both excitatory and inhibitory synapses (Woolfrey et al., 2009). Interestingly EPAC2 has been demonstrated to be important for excitatory transmission (Woolfrey et al., 2009; Yang et al., 2012) and has been shown to influence inhibitory transmission in dopamine neurons of the ventral tegmental area (Tong et al., 2017). Therefore, we were interested in examining whether loss of EPAC2 would impact excitatory and inhibitory synapses on the same neuron. Wild-type or Epac2−/− neurons (DIV 25) were immunostained with antibodies against vesicular glutamate transporter 1 (VGluT1) and vesicular GABA transporter (VGAT), presynaptic markers for excitatory and inhibitory synapses, respectively (Fig. 5A). When we examined the linear density of these presynaptic markers along the dendrites of pyramidal neurons, we found a decrease in the ratio of VGluT1 to VGAT puncta in Epac2−/− compared to wild-type neurons (Fig. 5B). This effect appeared to be mediated not by any change in VGluT1 puncta density (Fig. 5C), but rather a significant increase in VGAT puncta (Fig. 5D). These data suggest that inhibitory synapse numbers are increased in the absence of Epac2.

3.5. Key synaptic proteins are enriched in synaptosomal fractions in Epac2−/− brains

Our ICC experiments in primary neuronal cultures indicate that Epac2−/− neurons have larger dendritic spines with a concurrent increase presence of GluA2/3-containing AMPA receptors and the adhesion proteins NL3 and N-cadherin. To confirm that these effects also occur in vivo, we examined the presence of these key synaptic proteins within crude synaptosomal fractions (P2) generated from Epac2−/− or Epac2−/− cortex. First, we assessed whether the expression of these proteins was altered between wildtype and knockout animals. Consistent with our previous work (Srivastava et al., 2012a), we did not detect any change in the expression of GluA2, NL3, N-cadherin or the
presynaptic markers VGAT or VGluT1 in whole cell lysates between genotypes (Fig. 6A, B). However, when we examined the presence of these synaptic proteins within P2 fractions, we found a significant increase in expression of all proteins assessed, except for VGluT1 (Fig. 6D, E). These data, taken together with our ICC data, indicate an increased enrichment of AMPA receptor subunits and adhesion proteins at synapses in Epac2 knockout brains.

3.6. Epac2 is required for establishment of normal synaptic and dendritic structures in the ACC

We have previously shown that loss of Epac2 alters the dendritic organization and spine dynamics of layer 2/3 and layer 5 neurons, respectively, located in pre-motor and somatosensory areas (Srivastava et al., 2012a; Srivastava et al., 2012b). Moreover, EPAC2 has been shown to be required for maintaining spine morphology as well as density in vitro and in vivo (Srivastava et al., 2012a; Woolfrey et al., 2009). As the analysis of EPAC2 effects on spine and dendritic morphologies had thus far been limited to the pre-motor and somatosensory areas, we were interested whether loss of Epac2 also impacted these parameters in neurons from another cortical region. As Epac2−/− mice display gross disorganization of the ACC (Srivastava et al., 2012a), we therefore focused on the synaptic and dendritic morphologies of layer 5 neurons in this cortical region.

First, we assessed the linear density of spines on apical and basal dendrites of layer 5 neurons from the ACC of Epac2+/+GFP and Epac2−/− mice (Fig. 2). Loss of EPAC2 increases the colocalization of PSD-95 and GluA2/3 in dendrites. (A) Confocal images of cultured cortical neurons (DIV 22) from wild-type or Epac2−/− mice, immunostained for PSD-95 and GluA2/3. Scale bars = 5 μm. (B) Quantification of linear density of PSD-95 puncta in neurons in panel A. (C) Quantification of GluA2/3 puncta density and comparison of genotypes reveals increased density of GluA2/3 puncta. *P = 0.0277, Student’s t-test; n = 8–11 cells per condition, 3 independent cultures/genotype. (D) Quantification average cluster intensity and comparison of genotypes of GluA2/3 puncta. *P = 0.0112; Student’s t-test; n = 8–11 cells per condition, 3 independent cultures/genotype. (E) Quantification of colocalization, as measured by percentage of PSD-95 puncta containing GluA2/3 immunofluorescence signal, in wild-type and Epac2−/− neurons. **P = 0.0066, Student’s t-test; n = 8–11 cells per condition, 3 independent cultures/genotype. Data are presented as Box and Wisker plots with error bars showing minimum and maximum data points.
−GFP mice. This analysis revealed no difference in the density of dendritic spines along apical or basal dendrites of layer 5 neurons in the ACC (Fig. 7A–B). Analysis of spine morphology revealed that spine on apical dendrites of layer 5 ACC neurons in Epac2−/−GFP mice had significantly larger spine areas; there was no difference in spine area of spines on basal dendrites between wildtype and knockout animals (Fig. 7A–C). These data provide further evidence that Epac2 regulates spine stability in vivo.

Next, we examined the dendritic architecture of layer 5 ACC neurons in wildtype and Epac2 knockout mice. As described for layer 2/3 neurons in the somatosensory cortex (Srivastava et al., 2012b), layer 5 ACC neurons had a reduced number of basal, but not apical, dendrites (Fig. 8A and B). Interestingly, both apical and basal dendrite branches were on average significantly longer in neurons from Epac2−/−GFP mice (Fig. 8C). Consistent with these abnormalities, assessment of branch complexity as a function of branching order, revealed that both apical and basal higher order branch number were significantly reduced Epac2−/−GFP mice versus wild-type mice (Fig. 8D). These data are consistent with previous work demonstrating a role for EPAC2 in controlling the development of dendritic arborization.

4. Discussion

EPAC2 is a major PKA-independent target for cAMP in the mammalian forebrain. Through its ability to regulate the small GTPase Rap1, EPAC2 is involved in regulating synapse stability (Woollfrey et al., 2009). In addition, EPAC2 is required for the establishment of basal dendritic arborization of layer 2/3 neurons in vivo (Srivastava et al., 2012b). Multiple studies have also shown that EPAC proteins are required for normal cognitive functions (Yang et al., 2012) with EPAC2 being required specifically for socio-communicative behaviors (Srivastava et al., 2012a) as well as playing a role in controlling anxious
and depressive behaviors (Zhou et al., 2016). However, a comprehensive understanding of the role for EPAC2 in the development of the brain and organization of synapses is not fully understood. In this study, we confirm that EPAC2 is required for cAMP-mediated changes in spine morphology. Furthermore, we show that loss of EPAC2 results in the increased expression of GluA2/3-containing AMPA receptors and the adhesion protein N-cadherin at synapses. Interestingly, neurons from Epac2−/− also appear to have an increase in inhibitory synaptic markers with no change in the density of excitatory synaptic markers. Finally, we find that EPAC2 loss results in alterations in spine morphology and development of dendritic arborization of layer 5 ACC neurons in vivo. These data provide further evidence that expression of EPAC2 is required for the normal development of dendritic architecture, and moreover, is a critical regulator of synapse organization, particularly in the establishment of synapse stability and the ratio of excitatory and inhibitory synapses.

Analysis of cultured cortical neurons revealed that neurons from Epac2−/− mice had larger spine areas. This is consistent with previous work that has shown that EPAC2 is involved in regulating spine stability and motility (Srivastava et al., 2012a; Woolfrey et al., 2009). Larger spine would be more stable, have reduced motility and thus likely have altered responses to stimuli that would induce changes in spine morphology, ultimately impacting the ability of neural circuitry to respond to plasticity inducing stimuli (Kasai et al., 2010; Penzes et al., 2011). Interestingly, although we did not observe a change in the expression of NL3, a binding partner of EPAC2 (Woolfrey et al., 2009), we did observe an increase in the enrichment of this adhesion protein at synapses in Epac2 knockout cultures and in vivo. Concurrent with this, we also

Fig. 4. Loss of EPAC2 increases the colocalization of PSD-95 and N-cadherin in dendrites. (A) Confocal images of cortical neurons from wild-type or Epac2−/− mice (DIV 22), immunostained for PSD-95 and N-cadherin (N-cad). Scale bar = 5 μm. (B–C) Quantification and comparison of PSD-95 (B) and N-cadherin (C) puncta density between genotypes (Student t-test). (D). Quantification of N-cadherin puncta intensity revealed that average cluster size was larger in Epac2−/− neurons. ***P < 0.001, Student’s t-test; n = 12–16 cells from 3 experiments/genotype. (E) Quantification of colocalization, as measured by the percentage of PSD-95 puncta that also contained N-cadherin immunofluorescence signal. Genotypes were compared by Student’s t-test; **P = 0.0056; n = 12–16 cells from 3 experiments/genotype. Data are presented as Box and Wisker plots with error bars showing minimum and maximum data points.
observed an increase in the size of the pre-synaptic active zone marker bassoon. This may indicate that in addition to an increased number of larger dendritic spines, pre-synapses are also enlarged. Moreover, concurrent with an increase in spine size, we also observed an increase in the size of N-cadherin puncta at synapses in Epac2 knockout cultures. An increase in N-cadherin at synapses has previously been shown to be linked with larger, more stable spines (Mendez et al., 2010; Xie et al., 2008). Thus, an increase in the amount of N-cadherin at synapses would be in line with larger spines more stable spines.

Previous work has shown that EPAC2 activation decreases synaptic expression of GluA2/3 and AMPA-mediated transmission (Woolfrey et al., 2009). Consistent with these results, we found that Epac2−/− neurons had an increased density of GluA2/3-containing AMPA receptors, specifically at synapses. EPAC proteins and EPAC2 have been shown to be required for cAMP-dependent long-term depression (LTD) as well as cocaine-induced switching of AMPA receptor subunit composition (Liu et al., 2016; Ster et al., 2009). The consequence of increased synaptic expression of GluA2/3-containing AMPA receptors would potentially impact the ability of neurons to undergo plasticity-induced functional changes. Such deficits would also be consistent with our observation that loss of EPAC2 causes the formation of larger and more stable dendritic spines. Our data indicates that there is an increased presence of GluA2/3-containing AMPA receptors both in vitro and in vivo. Thus, EPAC2 appears to be required for maintaining the ability of neurons to undergo destabilization.

Epac2−/− neurons also exhibited an increase in VGAT puncta, suggesting an increase of inhibitory synaptic input onto these neurons concurrent with increased synaptic glutamate receptor content. It may be somewhat surprising that an increase in GluA2/3 puncta is not accompanied by an increase in VGluT1 puncta density. But this result may

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**Fig. 5.** Loss of EPAC2 decreases the ratio of excitatory (VGluT1) to inhibitory (VGAT) synaptic markers along dendrites. (A) Representative confocal images of cortical neurons from wild-type or Epac2−/− mice (DIV 25), immunostained for VGluT1 and VGAT. Images were obtained by confocal microscopy. Scale bar = 5 μm. (B) Quantification of the ratio of VGluT1 puncta density to VGAT puncta density. Genotypes were compared by Student’s t-test, *P = 0.0108; n = 14–16 cells from 4 independent culture/genotype. (C-D) Quantification of VGluT1 puncta density. Genotypes were compared by Student’s t-test, *P = 0.0276; n = 14–16 cells from 4 independent culture/genotype. Data are presented as Box and Wisker plots with error bars showing minimum and maximum data points.
be explained by the fact that PSD-95 puncta numbers are not changing, indicating that excitatory synapse numbers are similar in the presence and absence of EPAC2. Taken together, these data support a model in which the absence of EPAC2 leads to over-stabilized excitatory synapses, which also leads to an increase in the number of inhibitory inputs as a homeostatic response to the likely strong glutamatergic transmission occurring at these over-stabilized synapses.

An interesting observation in this study is that layer 5 neurons located in the ACC exhibit subtle changes in both dendritic and synaptic structures. Knockdown of EPAC2 in vivo results in the loss of dendritic spines on apical and basal dendrites of layer 2/3 neurons (Srivastava et al., 2012b). In contrast, no change in spine density was observed on layer 5 neurons in the ACC, but an increase in the number of spines with a larger area were found on apical dendrites. Similar to what we have...
previously reported following knockdown of EPAC2 on layer 2/3 neurons (Srivastava et al., 2012b), Epac2−/− mice had reduced basal dendrite number and complexity on layer 5 neurons in the ACC. Interestingly, we also observed subtle alteration in the length of dendritic branches on both apical and basal dendrites of these cells. It is likely that the more pronounced effect of EPAC2 loss on basal dendrites is due to the asymmetrical distribution of the EPAC2 protein throughout the dendritic tree. Moreover, the EPAC2-dependent regulation of basal dendrites appears to be due to EPAC2’s ability to directly regulate Ras signaling (Srivastava et al., 2012b). Moreover, recent evidence suggests that EPAC2 complexes with the Rac GEF kalirin (Wilkinson et al., 2017). Therefore, in future studies it will be interesting to see whether Ras or Rac signaling may be perturbed in Epac2−/− mice.

The Epac2 gene and its protein product have been implicated in a number of psychiatric disorders. Epac2 is located in the 2q31-q32 region, which was identified by several genome-wide linkage studies as an important autism susceptibility locus (Buxbaum et al., 2001; Shao et al., 2002). Microdeletion of the 2q31.1 region has been associated with intellectual disability and developmental delay (Williams et al., 2010). Recent studies also identified several copy number variants (CNVs) in this region in patients with autism, as well as enrichments of CNVs disrupting genes involved in GTPase/Ras signaling in autistic patients (Marshall et al., 2008; Pinto et al., 2010). Several rare mutations in the Epac2 gene have been identified in subjects with autism (Bacchelli et al., 2003), and interestingly, several of the mutations altered protein function, spine morphology and dendritic architecture (Srivastava et al., 2012b; Woolfrey et al., 2009). Because abnormal social and communication behavior is characteristic of a number of neurodevelopmental and neuropsychiatric disorders, and that Epac2 knockout mice show impaired socio-communicative behaviors (Srivastava et al., 2012a; Yang et al., 2012), gaining a greater understanding of EPAC2 function in vivo may provide further insight into the

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pathogenesis of these diseases.

Acknowledgments

This work was supported by NIH grants R01MH071316 and R01MH097216 to P.P.; and NRSA USA Ruth L. Kirschstein Award F31MH085362 to K. A. J.; and grants from the Medical Research Council UK, MR/L021064/1, Royal Society UK (Grant RG130856), and the Brain and Behavior Foundation (formally National Alliance for Research on Schizophrenia and Depression (NARSAD); Grant No. 25957), awarded to D.P.S.

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