Local CRH Signaling Promotes Synaptogenesis and Circuit Integration of Adult-Born Neurons

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SUMMARY

Neural activity either enhances or impairs de novo synaptogenesis and circuit integration of neurons, but how this activity is mechanistically relayed in the adult brain is largely unknown. Neuropeptide-expressing interneurons are widespread throughout the brain and are key candidates for conveying neural activity downstream via neuromodulatory pathways that are distinct from classical neurotransmission. With the goal of identifying signaling mechanisms that underlie neuronal circuit integration in the adult brain, we have virally traced local corticotropin-releasing hormone (CRH)-expressing inhibitory interneurons with extensive presynaptic inputs onto new neurons that are continuously integrated into the adult rodent olfactory bulb. Local CRH signaling onto adult-born neurons promotes and/or stabilizes chemical synapses in the olfactory bulb, revealing a neuromodulatory mechanism for continued circuit plasticity, synapse formation, and integration of new neurons in the adult brain.

INTRODUCTION

Synaptogenesis and circuit integration of neurons in the brain is governed by diverse repertoires of synaptic and extrasynaptic inputs. Excitatory input by principal neurons has profound effects on sculpting and pruning synaptic connectivity (Favero and Castro-Alamancos, 2013; Le Roux et al., 2013). However, recent evidence suggests that interneurons also play significant roles in modulating synapse formation (De Marco García et al., 2011; Hensch et al., 1998; Le Magueresse and Monyer, 2013). Inhibitory interneurons are highly heterogeneous and, depending on the brain region, can vastly outnumber principal neurons (Chen and Greer, 2004; Isaacson and Strowbridge, 1998; Lledo et al., 2008). Neurochemical classification schemes have shown that interneurons not only express GABA and calcium-binding proteins such as parvalbumin (PV), calretinin (CR), and calbindin (Barinka et al., 2012; Kosaka and Kosaka, 2008; Rudy et al., 2011), but also a cast of neuromodulatory peptides, including somatostatin (SST), cholecystokinin, oxytocin, and corticotropin-releasing hormone (CRH) (Huang et al., 2013; Le Magueresse and Monyer, 2013; Ma et al., 2006; Rudy et al., 2011; Xu et al., 2013). Neuropeptidergic interneurons are promising candidates for modulating changes in local synapse and circuit structure, and are pervasive throughout the rodent olfactory bulb (OB).

Endowed with the feature of ongoing neurogenesis (Alvarez-Buylla and Temple, 1996), the mouse olfactory system offers an excellent in vivo model to investigate mechanisms that underlie synaptogenesis, circuit plasticity, and the integration of new neurons into existing networks (Abrous et al., 2005; Ming and Song, 2005). Adult-born neurons are continuously generated in the subventricular zone (SVZ), migrate via the rostral migratory stream (RMS), and populate the OB where the vast majority become inhibitory granule cells that form connections with OB principal mitral and tufted cells (Mori et al., 1983; Price and Powell, 1970a, 1970b; Carleton et al., 2003; Shepherd and Greer, 2004). This interaction influences olfactory behaviors and odor-related memories (Abraham et al., 2010; Breton-Provencher et al., 2009; Mouret et al., 2009; Rochefort et al., 2002).

Studies have found that the survival and integration of adult-born granule cells is activity-dependent during a developmental critical period between 2 and 4 weeks after their birth (Kelsch et al., 2009; Yamaguchi and Mori, 2005), when they receive inputs from local interneuron subtypes, including deep and superficial short axon cells and Blnanes cells (Arenkiel et al., 2011; Eyre et al., 2008; Pressler and Strowbridge, 2006), as well as centrifugal fibers from deeper regions of the brain (Arenkiel et al., 2011; Balu et al., 2007; Panzanelli et al., 2009; Whitman and Greer, 2007). Maturing granule cells extend their dendrites into the external plexiform layer (EPL), where they connect with principal mitral cells. Interestingly, the EPL also harbors a more dispersed, and heterogeneous population of neuropeptidergic interneurons (Kosaka and Kosaka, 2008; Lepousez et al., 2010a, 2010b) that also form reciprocal synaptic connectivity with mitral cells (Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013). Unlike granule cells, EPL interneurons are generated in the early postnatal period and remain stable throughout life (Batista-Brito...
### Developmental Cell
Neuropeptide Signaling and Circuit Integration

#### Figure A

A diagram illustrating the experimental setup involving Gene Regulatory Systems (GREG) and the use of Cre recombinase.

#### Figure B

A photomicrograph showing the expression of SADΔG-EGFP RV in the OB.

#### Figure C

A diagram illustrating the expression pattern of tdTomato/SADΔG-EGFP RV in the OB.

#### Table D

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- **SADΔG-EGFP RV**
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et al., 2008), but their potential neuromodulatory role in shaping the integration of adult-born neurons is unknown.

We have previously shown that odor enrichment increases the number of inputs onto adult-born neurons in the OB (Arenkiel et al., 2011), causing enhanced cell survival and integration. However, the precise signaling mechanisms between these inputs and granule cells remain in question. In this study, we have mapped local neuropeptidergic EPL interneurons with anatomical and functional connectivity onto granule cells during peak periods of synaptogenesis and circuit integration. Using loss- and gain-of-function analyses, conditional viral-genetic technologies, optical imaging, electrophysiological recordings, and optogenetic stimulation, we have uncovered a neuropeptidergic signaling mechanism between local CRH+ EPL interneurons and adult-born granule cells that plays an important role in synapse formation, circuit plasticity, and the integration of new neurons into the existing networks, revealing a dual functional role for neuropeptidergic inhibitory interneurons in the mouse OB.

RESULTS

Adult-Born Neurons Receive Inputs from Local EPL Interneurons

To reveal the identities of the presynaptic inputs made onto adult-born granule cells, we performed targeted monosynaptic tracing using genetically engineered rabies virus (RV), SADG-EGFP RV (Arenkiel et al., 2011; Wickersham et al., 2007a, 2007b). RV is a neurotropic virus that travels retrogradely between connected neurons. Endowing it with the avian coat protein EnvA provides selectivity of infection to source cells that express the virus, making it a useful tool for tracing the connectivity of neural circuits. RV binding in mitral cells (Figure 1D), validating RV jumping to known synaptic partners. Interestingly, we found that adult-born granule cells also received extensive inputs from both deep and superficially located interneurons, with highest enrichment from cells located in the EPL (Figure 1D).

Local CRH+ EPL Interneurons Make Connections onto Adult-Born Granule Cells

Immunohistochemical characterization of the presynaptic EGFP+ EPL cells revealed that granule cell inputs constituted a subset of nondopaminergic interneurons (GFAP, 0%; tyrosine hydroxylase, 0%; CR+, 92% ± 5%; Figures S1B–S1D), with strong colabeling of PV (95% ± 3%; Figure 1E), and SST (45% ± 5%; Figure S1E). Highest neuropeptide expression was observed with CRH (68% ± 4%; Figure 1F), suggesting that adult-born neurons received direct input from resident CRH+ interneurons in the OB. Although immunohistochemistry identified enrichment of CRH protein in EPL interneurons, we also detected substantial extracellular CRH in the EPL (Figure 1F), suggesting that CRH is locally secreted. To more precisely identify CRH+ interneurons as bona fide inputs, we used genetic labeling techniques.

To this end, we crossed CRH-Cre mice to conditionally targeted tdTomato reporter mice (CRH-Cre+/-; ROSA<sup>LSL-tdTom</sup>/C0), and observed strong tdTomato signal in the paraventricular nucleus of the hypothalamus, a known hub for CRH synthesis and secretion (Figure S2), and high levels of expression in EPL interneurons of the OB (Figure 2A), which we previously characterized as multipolar and anaxonic fast-spiking PV+ interneurons (Huang et al., 2013). To determine if adult-born granule cells received inputs from CRH+ EPL interneurons, we performed transsynaptic tracing in CRH-Cre<sup>+/-</sup>; ROSA<sup>LSL-tdTom</sup>/C0, and electropropated the avian TVA receptor and rabies-G glycoprotein into the SVZ of CRH-Cre<sup>+</sup>; ROSA<sup>LSL-tdTom</sup>-/pups (Figure 2B), transiently targeting neural progenitors that give rise to adult-born neurons. 28 days later, mice were injected with RV into the core of the OB, and killed 7 days later. OB slices revealed strong EGFP expression, with high efficiency injection of targeted granule source cells and presynaptic inputs (Figure 2C). Both source cells and all inputs were labeled EGFP+, whereas CRH+ inputs were EGFP+/tdTomato+. Through this differential labeling, we verified that new granule cells indeed received extensive input from local CRH+ EPL interneurons with 86% (±5% SEM) of EPL presynaptic inputs expressing CRH via lineage tracing.
Adult-Born Granule Cells Dynamically Express CRHR1

CRH is best known as a hypothalamic regulatory hormone that mediates systemic stress responses (Vale et al., 1981; Vale et al., 1983). In addition, CRH has been implicated both as a neurotransmitter and neuromodulator in the hippocampus, amygdala, and cerebellum (Maras and Baram, 2012; Rozendaal et al., 2008; Schmolesky et al., 2007). CRH can bind to two G protein coupled receptors, CRHR1 and CRHR2 (Perrin et al., 1993, 1995; Perrin and Vale, 1999), but in the brain shows higher affinity to CRHR1, which mediates many of its physiological effects (Bale and Vale, 2004). When bound to CRHR1, Gs-coupled signaling is activated (Berger et al., 2006; Blank et al., 2003; Perrin et al., 1993; Thiel and Cibelli, 1999).

Having identified that CRH+ EPL interneurons provide inputs onto granule cells, we next sought to investigate the expression of CRH receptors in the OB. RT-PCR revealed that both CRH and CRHR mRNAs were present in the bulb, and that CRHR1 was expressed at much higher levels than CRHR2 (Figure 3A). Because available antibodies for CRHR are not useful for detecting endogenous CRHR1 (Refojo et al., 2011) and the expression pattern is not conclusive in the OB (Figure S3A), we used genetic strategies to determine the CRHR1 cell type-specific expression pattern. Consistent with mRNA transcript detection, tissue sections from CRHR1-EGFP BAC transgenic mice, whose expression pattern was previously validated to match endogenous CRHR1 (Justice et al., 2008), revealed high levels of spatially restricted CRHR1 in granule cells (Figure 3B). Finally, to determine the precise spatial localization of CRH and CRHR1 neurons within the OB, CRHR1-EGFP mice were crossed to CRH-Cre−/−; ROSALSL-tdTom−/− mice to generate CRHR1-EGFP+/−; CRH-Cre+/−; ROSALSL-tdTom+/− double reporter mice. OB sections showed a cell type-specific juxtaposition between EGFP-labeled CRHR1+ granule cells, and tdTomato-labeled CRH+ EPL interneurons (Figure 3C). Whereas CRHR1+ granule cell bodies were located throughout the GCL with superficial enrichment, CRH+ EPL interneurons almost exclusively resided in the EPL, were absent from the GCL (Figure S3B), and directly juxtaposed CRHR1+ dendrites. These data support the idea that CRH ligand is locally released by EPL interneurons and directly acts on CRHR1+ granule cell dendrites in the EPL.

EGFP expression was nearly absent from the RMS and became gradually enriched in the outer GCL, with strong enrichment in superficial regions (Figures 3B and 3C). These data suggested that CRHR1 might exhibit a dynamic expression pattern with granule cell maturation, and that CRHR1 might be expressed both in early postnatal-born granule cells which predominantly localize to the superficial GCL (Lemasson et al., 2005), as well as adult-born granule cells. To test if CRHR1 is dynamically regulated during periods of newborn granule cell synaptogenesis and to better determine its spatiotemporal expression profile, CRHR1-EGFP mice were pulsed with the proliferation marker EdU, and killed between 7 and 60 days, spanning early, intermediate, and late phases of synaptogenesis and circuit integration (Figure 3D; Carleton et al., 2003). We...
Figure 3. Adult-Born Granule Cells Dynamically Express CRHR1

(A) Semiquantitative RT-PCR for CRH and CRHR1/2 of whole OB RNA.

(B) OB cross-section of CRHR1-EGFP BAC transgenic mice. RMS, rostral migratory stream; GCL, granule cell layer; EPL, external plexiform layer; GL, glomerular layer; scale bar represents 200 μm.

(C) Reporter expression of CRHR1-EGFP; CRH-Cre<sup>+/−</sup>; ROSA<sup>tdTom</sup> mice (arrows point to CRHR1+ granule cells; open arrowheads mark CRH+ EPL interneurons; scale bars represent 60 and 20 μm).

(D) Experimental scheme to determine the developmental expression profile of CRHR1 expression in granule cells.

(E) CRHR1 expression in newborn neurons 28 days post EdU injection (scale bar represents 60 μm).

(F) Quantification of CRHR1-expression in granule cells (data points represent averages ± SEM, n = 3 animals per time point).

(G) CRHR1::EGFP expression in adult-born granule cells (scale bars represent 100 and 20 μm).

See also Figure S3.
found that the ratio of EdU-labeled granule cells that expressed CRHR1 was very low at 7 days of age (5.8% ± 3.9%; Figure 3F), and substantially increased between 14 (34.5% ± 4.1%), 21 (59.2% ± 4.2%), and 28 days of age (81.3% ± 2.2%). This value slightly increased further at 40 days post-EdU pulsing (87.8% ± 4.5%), and the number of colabeled neurons plateaued at 60 days (87.5% ± 3.2%). Intriguingly, dynamic enrichment of CRHR1 coincided with critical periods of activity-dependent survival, synaptogenesis, and circuit integration between 14 and 28 days of granule cell age (Kelsch et al., 2009; Mouret et al., 2008; Yamaguchi and Mori, 2005).

Finally, to determine the subcellular localization of CRHR1, we expressed a CRHR1::EGFP fusion construct with a tdTomato cell fill in new granule cells. We observed CRHR1::EGFP in dendrites, with enriched subcellular localization in a subset of dendritic spines in the EPL (Figure 3G). Interestingly, CRHR1::EGFP was also present in extrasynaptic dendritic regions (Figures S3C–S3E), suggesting that CRH-mediated local neuropeptide signaling might not occur exclusively at synapses, but also via extrasynaptic mechanisms not restricted to dendritic spine structures.

Together, these data support the idea that CRH is synthesized locally by EPL interneurons and can signal to granule cells via time-dependent expression of CRHR1, suggesting a possible role for secreted CRH in the long-term survival and circuit integration of adult-born neurons.

**CRH Signaling Is Required for Normal Levels of Adult-Born Granule Cell Survival**

To determine how CRH signaling affects adult-born neurons, mice lacking CRH or its receptor (CRH<sup>−/−</sup> or CRHR1<sup>−/−</sup>) were pulsed with bromodeoxyuridine (BrdU) and killed either 24 hr later to assay proliferation in the SVZ (BrdU+/Ki67+ cells), or 30 days later to assay survival in the GCL (BrdU+ cells; Figure 4A), focusing on deeper regions where adult-born granule cells reside (Lemasson et al., 2005). Compared to wild-type littermates, CRH<sup>−/−</sup> or CRHR1<sup>−/−</sup> mutants showed increased SVZ proliferation (p < 0.05, 4,942 ± 302 cells in CRH<sup>−/−</sup> mice, compared to 3,906 ± 354 cells in CRH<sup>+/+</sup> mice), but decreased cell survival in the OB (p < 0.005, 25 ± 1 cell in CRH<sup>−/−</sup>, compared to 36 ± 2 cells in CRH<sup>+/+</sup> mice; Figures 4B and C). This increased proliferation in CRH<sup>−/−</sup> mice is consistent with previous reports that stress impairs neurogenesis both in the SVZ and in the hippocampus (de Andrade et al., 2013; Hitoshi et al., 2007, Schoenfeld and Gould, 2013), and that CRH<sup>−/−</sup> mice show decreased stress levels (Jacobson et al., 2000). Cleaved caspase-3 and TUNEL staining revealed increased apoptosis in the GCL of CRH<sup>−/−</sup> mice (Figure S4A). To investigate whether granule cell apoptosis was secondary to loss of CRH+ interneurons, we examined the integrity of the EPL and performed cell counts using DAPI and CR, which overlaps with EPL interneurons (Huang et al., 2013; Figures S1D, S4B, and S4C) and found no difference. Moreover, because CRH has important systemic effects as a regulatory hormone, many of which are mediated by corticosteroids, we questioned whether the attrition of granule cells in CRH<sup>−/−</sup> mice was corticosteroid-dependent and supplemented adult CRH<sup>−/−</sup> mice with corticosterone. Corticosteroid supplementation at a concentration that readily crosses the blood-brain barrier and is capable of rescuing embryonic phenotypes in utero (Muglia et al., 1995), did not change granule cell survival in CRH<sup>−/−</sup> mice (Figure S4F), suggesting that adult-born neuron survival was not mediated by systemic CRH signaling, but likely through local CRH.

As loss of secreted CRH systemically in CRH<sup>−/−</sup> mice could have secondary effects in the OB, we next assayed for proliferation and cell survival in CRHR1<sup>−/−</sup> mice, and saw no change in SVZ-based proliferation compared to control littermates (p > 0.05, 4,172 ± 113 cells in CRHR1<sup>−/−</sup> versus 4,337 ± 303 cells in CRHR1<sup>+/+</sup> mice, Figure 4D). These data were consistent with the observation that the SVZ lacked CRHR1 expression in CRHR1::EGFP mice (data not shown), and that SVZ proliferation might be mediated by systemic signaling rather than through central CRH receptor activation. However, decreased numbers of granule cells might be noted in the OB 30 days post-BrdU pulsing (p < 0.001, 24 ± 1 cells in CRHR1<sup>−/−</sup> versus 32 ± 2 cells in CRHR1<sup>+/+</sup> mice, Figure 4E), as well as increased numbers of apoptotic cells (Figure 4F). Moreover, this phenotype was also corticosteroid-independent (Figure S4G).

To bypass any potential systemic effects of using germline CRH loss-of-function alleles, we next conditionally removed CRHR1 specifically from adult-born granule cells by injecting a mixture of equal titers of AAV particles that expressed either Cre-P2A-EGFP or tdTomato (control) into the RMS of CRHR1<sup>+/+</sup> or CRHR1<sup>flox/flox</sup> mice (Kühne et al., 2012), and revealed a 24.8% ± 2.8% decrease in the ratio of Cre-EGFP/+ tdTom+ granule cells between CRHR1<sup>flox/flox</sup> and CRHR1<sup>flox/flox</sup> mice (Figures 4F and 4G). Interestingly, morphological analysis on the proportion of surviving granule cells showed no difference between Cre and tdTomato+ neurons (data not shown).

Having found decreased numbers of surviving granule cells in CRH loss-of-function mutants, we wondered if synaptic protein expression was affected in these models and performed western blot analysis, which showed significantly decreased levels of Synapsin, PSD95, and NR2B in the OBs of CRH<sup>−/−</sup>,
CRHR−/−, and CRHR1floxp/floxp mice compared to controls (Figures 4H–4P). Together, these data imply an important role for CRH signaling in the maintenance and/or generation of synapses in the OB.

We next asked if CRHR1 expression correlates with cell survival, and stained CRHR1-EGFP olfactory bulb tissue with activated caspase-3 (Figure S4H). Interestingly, we did not observe any CRHR1-EGFP+/caspase-3+ cells. Together, these loss-of-function and molecular marker data suggest that granule cells require local CRH signaling for normal development and maturation, and that CRHR1 expression correlates with synapse formation and/or survival, allowing granule cells to integrate into existing brain circuits.

**Gain-of-Function CRH Signaling Promotes Increased Synaptic Protein Expression in the OB**

Having found that lack of CRH signaling leads to impaired granule cell survival (Figure 4), we next queried the consequence of enhanced CRH signaling. To determine a role for CRHR1 in shaping synapse development and neuronal maturation, we expressed a constitutively active version of CRHR1 fused to EGFP, (CA)CRHR1:EGFP (Nielsen et al., 2000) in newborn granule cells (Figure 5A). Morphological characterization of granule cells expressing (CA)CRHR1:EGFP revealed normal average neuron length comparable to tdTomato controls (p > 0.05, 284 ± 21 μm in (CA)CRHR1:EGFP versus 316 ± 19 μm in controls, Figure 5B), but increased total dendrite length (p < 0.05, 894 ± 30 μm versus 672 ± 25 μm, Figure 5C), dendritic branch number (p < 0.01, 10.5 ± 0.65 m versus 7 ± 0.7 μm, Figure 5D), and branch intersections at both proximal and distal radii from the soma (p < 0.05; Figure 5E). Next, we quantified dendritic spines and found that total spine number was increased in (CA)CRHR1:EGFP neurons (Figure 5F). Neurolucida reconstructions revealed increased complexity in (CA)CRHR1:EGFP granule cells compared to control neurons (Figure 5G).

Then, we targeted granule cells in the OB for conditional gain-of-function studies with spatiotemporal specificity during periods of endogenous CRHR1 expression to determine if CRHR1 activation is sufficient for modulating synaptogenic changes. For this, we first generated a BAC transgenic allele to drive Cre recombinase from the CRHR1 promoter. To validate cell type specificity of Cre expression, we generated CRHR1-EGFP;CRHR1-Cre+/;ROSA26LysCre+ mice, and sawCre activity that matched the expression pattern of CRHR1-EGFP transgenic mice in the OB (Figure S5A). We next generated a conditional adeno-associated virus that carries (CA)

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(F) Quantification of the number of dendritic spines between tdTomato and (CA)CRHR+ granule cells (p < 0.05 ANOVA). N = 10 cells each from three animals. (G) Representative granule cell morphology reconstructions. (H) Experimental scheme for targeting CRHR1+ OB granule cells for constitutive CRHR1-activation. (I) Expression pattern of AAV-flex-(CA)CRHR-GFP in granule cells (arrows point to (CA)CRHR1::GFP+ neurons; scale bars represent 1,000, 100, and 20 μm). (J–O) Western blot analysis of synaptic protein expression of CRHR1-Cre+ OBs injected with either flexed GFP or flexed-(CA)CRHR1::GFP AAV (p < 0.05 Student’s t test, n = 4 animals each). All data points are averages ± SEM. See also Figure S5.
CRHR1::EGFP in an inverted “flexed” configuration (Atasoy et al., 2008) and stereotactically targeted the RMS for infection of granule cells in CRHR1-Cre+/− mice (Figures 5H and 5I). To confirm enhanced signaling through CRHR1, we isolated whole OBs for western blot analysis. Control lysate was obtained from CRHR1-Cre+/− mice injected with serotype-matched flexed GFP virus. Since CRHR1 is Gs-coupled and leads to activation of granule cells in the RMS (Atasoy et al., 2008) and stereotaxically targeted the RMS for infection of granule cells in CRHR1-Cre+/− mice (Figures 5H and 5I), we next assayed pCREB levels in (CA)CRHR1-expressing OBs (Figure 5J), which were significantly increased. Interestingly, we also observed significant changes in levels of synaptic protein expression, including a large increase in the synaptic protein Synapsin (Figure 5K), suggesting the formation of de novo synapses or the strengthening of existing synapses with enhanced CRHR1 signaling. Intriguingly, however, although the quantity of PSD95 showed an increased trend of expression, these changes were not statistically significant (Figure 5L). Because granule cells are GABAergic and form reciprocal dendrodendritic synapses with mitral cells (Mori et al., 1983; Panzanelli et al., 2009; Shepherd and Greer, 2004), we also assayed for changes in both inhibitory and excitatory receptor expression. The mitral cell-specific GABA-Ax1 (Whitman and Greer, 2007), as well as NR1, and NR2B receptor subunits showed increased expression (Figures 5M, 5N, and 5S), whereas levels of AMPA receptor subtypes were decreased compared to GFP controls (Figures 5O and 5S). Immunohistochemistry for the upregulated proteins revealed increased levels of NMDA and GABA receptor expression in the EPL of gain-of-function experiments compared to controls (Figures S5D–S5F). Intriguingly, increased NR1 and NR2B expression was localized to both (CA)CRHR1::EGFP+ as well as EGFP lacking dendritic structures, which could be due to the nature of the membrane-bound overexpression construct. For example, the fusion protein is not exclusively targeted to all synaptic structures within newborn granule cells, and its synaptic properties may in fact influence the formation of synaptic structures in a more widespread manner. Consistent with this, we also noted significant upregulation of GABA-Ax1, which is expressed by mitral/tufted cells in the OB, and is not expected to colocalize with (CA)CRHR1::EGFP structures. Together, these data suggest an overall increase in synaptic connectivity within the OB circuitry with constitutive CRHR1 signaling, which then also may lead to non-cell-autonomous and more widespread increases in synaptic protein expression.

Together, increased spine numbers, upregulated Synapsin and NMDA receptor expression, combined with decreased AMPA receptors suggests that CRH signaling in the OB promotes the formation of new immature synapses, and/or potential synaptic scaling of existing excitatory synapses (Turrigiano et al., 1998; Turrigiano and Nelson, 2004).

**Gain-of-Function CRH Signaling Promotes Functional Synaptogenesis in the OB**

A hallmark of bona fide circuit integration is functional synaptic connectivity. Having determined that constitutively active CRHR1 signaling leads to synaptogenic changes in the OB (Figure 5), we next queried the functional consequence of gain-of-function CRH signaling using electrophysiology. To determine if soluble CRH ligand influenced granule cell electrophysiological properties, we made whole cell recordings from CRHR1-EGFP+ granule cells while bath-applying CRH, which showed no change in the frequency of miniature excitatory postsynaptic currents (mEPSCs), but induced a significant decrease in amplitude (p < 0.05, 11.55 ± 0.72 pA before, and 8.8 ± 0.35 pA after CRH; Figures 6A–6D). Whole cell recordings from granule cells that expressed (CA)CRHR1 compared to EGFP controls showed no change in mEPSC frequency, but significantly decreased mEPSC amplitudes (p < 0.05, 7.73 ± 1.38 pA in (CA)CRHR1 versus 11.69 ± 1.27 pA in EGFP controls; Figures 6E–6H). These data were consistent with the observation that CRH gain-of-function signaling through activated CRHR1 led to decreased AMPA receptor levels (Figures 5O and 5S) and further suggested that upregulation of glutamatergic synapses via NMDA receptor expression likely reflects functionally silent or immature synapses. Worth noting, we did not observe any rapid changes in firing, passive membrane properties, or membrane potential following CRH application (data not shown), suggesting that in the OB CRH does not act directly as a neurotransmitter, but likely functions as a true neuromodulator.

With the observation that GABA-Ax1 receptor subunit expression increased with CRH signaling (Figure 5M), we next hypothesized that functional GABAergic synapses from granule cells onto mitral cells might be upregulated. To address this, we recorded miniature inhibitory postsynaptic currents (mIPSCs) from mitral cells in OBs where CRHR+ granule cells expressed (CA)CRHR1::EGFP, and found a significant increase in frequency of mIPSCs (p < 0.05, 2.21 ± 0.39 Hz in (CA)CRHR1 versus 1.17 ± 0.18 Hz in EGFP controls, Figures 6I–6L), suggesting the formation or stabilization of granule cell–mitral cell synapses or changes in presynaptic release properties. Hence, biochemical and electrophysiological evidence suggests that enhanced CRHR signaling in granule cells leads to increased functional synaptogenesis and circuit plasticity in the OB.

**Acute Optogenetic Activation of CRH** EPL Interneurons Promotes Release of CRH in the OB

CRH+ EPL interneurons make connections onto granule cells, which in concert dynamically express CRHR1 (Figures 1, 2, and 3). Lack of CRHR1 expression during a critical time window in granule cell maturation and circuit integration leads to decreased cell survival (Figure 4), and constitutively active CRHR1 enhances synapse formation and circuit integration of adult-born neurons (Figures 5 and 6). Together these data support a mechanism by which activity-induced release of CRH from EPL interneurons may influence granule cell synaptogenesis. We next questioned if manipulating the activity of CRH+ EPL interneurons directly and acutely could dynamically recapitulate the physiological effects observed with constitutive CRHR1 activation.

To activate CRH+ EPL interneurons with spatiotemporal specificity, CRH-Cre+− mice were crossed to ROSA4Sl-Crh2 mice to obtain CRH-Cre+−/−; ROSA4Sl-Crh2 mice that selectively expressed the light gated cation channel channelrhodopsin 2 (ChR2; Boyden et al., 2005; Nagel et al., 2003) in CRH+ EPL interneurons in the OB. From these mice, we made slices of the OB (Figure 7A) and hypothalamus (Figure S5), which were acutely
photostimulated ex vivo in small volumes of artificial cerebral spinal fluid (ACSF). Light-stimulated depolarization of CRH+ neurons led to CRH release in both hypothalamic control (Figure 7B) and OB slices (Figure 7C), whereas soluble CRH levels in light-stimulated controls were unchanged. Together, these data suggest that the targeted depolarization of CRH+ neurons elicited the release of stored CRH neuropeptide from EPL interneurons.

Finally, to determine the effects of CRH+ EPL interneuron activation on granule cell synapses in vivo, CRH-CreErt2+; ROSA26SL-CreR+/C0 mice were chronically implanted with fiberoptics directly over the olfactory bulb (Ung and Arenkiel, 2012) and photostimulated with blue laser light (Figure 7D). Acute in vivo photostimulation in awake and behaving mice led to enhanced olfactory bulb expression of pCREB (Figure 7E), recapitulating the effect seen in constitutive CRHR1 activation (Figure 5J). Western blots of stimulated animals showed significant upregulation of the synaptic proteins Synapsin, PSD95, and NR2B (Figures 7F–7H), suggesting a conserved synaptogenic effect of photostimulated release of CRH from EPL interneurons in vivo, which mirrored constitutively active CRHR1 signaling (Figure 5).

DISCUSSION

Neuropeptides, including CRH, have been implicated in a variety of neural processes, ranging from neuromodulation and dendritic outgrowth, to neuroprotection (Chen et al., 2004; Hanstein et al., 2008; Sheng et al., 2012). However, the precise role of CRH in the maturation and integration of granule cells in the OB has not been investigated. Here, we describe a neuropeptidergic function for inhibitory interneurons in shaping cell survival, synaptogenesis, and circuit integration of new neurons in the adult brain that is distinct from classical neurotransmitter signaling. Together, our data support a potential mechanism for a tripartite-like interaction between mitral cells, EPL interneurons, and granule cells (Figure 7I). We and others have recently shown that mitral cells and EPL interneurons exhibit reciprocal connectivity (Huang et al., 2013, Kato et al., 2013; Miyamichi et al., 2013). It is conceivable that neuronal activity via olfactory sensation is conveyed to mitral cells, which in turn stimulate EPL interneurons to both shape mitral cell output, and release soluble CRH. In this way, activity-dependent release of CRH, and subsequent reception by granule cells that express CRHR1 both synthetically and/or extrasynaptically, may function as a key mechanism toward modulating synaptogenesis and plasticity via neuropeptide signaling, linking activity to circuit integration via neuropeptidergic interneurons. Ultimately, this interaction has the potential to shape whole circuit activity and plasticity. Although this model supports a role for local interactions, it remains to be determined if other forms of neuromodulatory signals are conveyed specifically to EPL interneurons via other local or centrifugal inputs.

Dual Roles of Interneurons

Neuropeptides are traditionally acknowledged for roles in shaping whole-body physiological responses and/or modulating systemic homeostatic mechanisms (Vale et al., 1981). Recently, neuropeptides have received increasing attention for their roles in shaping synapses and facilitating neuronal plasticity (Bayatti et al., 2003; Fenoglio et al., 2006; Lipschitz et al., 2005). Interestingly, however, interneurons have been most extensively studied with respect to their traditional, GABAergic inhibitory function, and the neuropeptides they express have served primarily in categorizing the many different interneuron subtypes throughout the brain (Ma et al., 2006; Rudy et al., 2011; Xu et al., 2013). Although granule cells make up the majority of inhibitory interneurons in the OB, previous work from our lab and others has identified other types of interneurons with GABAergic connections onto mitral cells that likely serve important olfactory functions (Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013; Lepousez et al., 2010a, 2010b; Kosaka and Kosaka, 2008). Here, we have identified a population of inhibitory interneurons in the OB with a neuropeptidergic role in promoting synaptic protein expression and circuit integration. Interestingly, this interneuron population has a dual role in shaping the OB circuitry and likely, olfaction. This interaction is inhibitory onto mitral cells and neromodulatory onto granule cells, suggesting increased connectivity and/or signaling not only between excitatory principal cells and inhibitory interneurons, but also between different subtypes of interneurons. This apparent dual functionality may indeed exemplify a prominent mechanism to support neuronal plasticity, and a way to shape circuitries by inhibitory neurotransmitter signaling onto mitral cells, and neromodulatory signaling onto granule cells. EPL interneurons form reciprocal GABAergic connections with mitral cells, and are in turn depolarized following odor stimulation (Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013). This activation further strengthens mitral cell inhibition, but may also facilitate CRH release, linking olfactory-dependent activity to neumodulation, and ultimately synaptogenesis and circuit integration.

Local Neuropeptide Signaling, Synaptogenesis, and Circuit Integration

Our data suggest that local neuromodulatory signaling by CRH+ EPL interneurons in the OB aids in granule cell circuit integration...
Figure 7. Optogenetic Activation of CRH+ EPL Interneurons Induces Synaptogenesis in the OB

(A) ChR2 expression pattern of CRH-Cre+; ROSA26LSL-CRHR1+/- mice (scale bars represent 200 and 50 μm).

(B and C) Quantification of CRH concentration with hypothalamic or OB optogenetic activation in ROSA26LSL-CRHR1+/- (control) or CRH-Cre+; ROSA26LSL-CRHR1+/- mice (*p < 0.05 Student’s t test, n = 3 animals per group).

(D) Experimental scheme for in vivo photostimulation of CRH+ EPL interneurons.

(legend continued on next page)
by increasing synaptic protein expression and/or stabilizing existing synapses. Removing CRH signaling decreases synaptic protein expression, whereas enhancing CRH signaling induces synaptic protein expression in the OB. Perhaps more intriguingly, the result that CRHR loss-of-function does not completely abolish adult-born neuron survival and integration poses some additional questions. Given that numerous neuropeptides, including aomatostatin, vasointestinal peptide, cholecystokinin, oxytocin, and neuropeptide Y are present in the olfactory bulb (Gracia-Llanes et al., 2003; Lepousez et al., 2010a, 2010b; Ma et al., 2006; Tobin et al., 2010), and that granule cells in turn appear to express the cognate receptors, it remains to be seen if other neuropeptidases have the same effect on the OB circuitry, and if these neuropeptides act in concert to promote synaptogenesis and circuit integration in the adult brain.

EXPERIMENTAL PROCEDURES

Experimental Animals

All experimental animals were treated in compliance with the United States Department of Health and Human Services and the Baylor College of Medicine IACUC guidelines. ROSA26R-TOMA/RTVA (Takahashi et al., 2013), ROSA26R-TOMA/RTVA (Arenkel et al., 2011), CRHR1–EGFP mice (Justice et al., 2008), and CRHR1–/– mice (Kühne et al., 2012) were previously described. CRH-Cre (Taniguchi et al., 2011), ROSA26R-tdTom (Madisen et al., 2012), CRH–/– (Muglia et al., 1995), and CRHR1–/– (Smith et al., 1998) were obtained from Jackson Laboratories and maintained on a C57BL/6 background. Generation of the CRHR1–Cre line is described in the Supplemental Experimental Procedures.

Transsynaptic Tracing

Briefly, adult (6–8 weeks old) ROSA26R-TOMA/RTVA mice were injected with high titer lentivirus encoding tdTomato-IRES-Cre stereotaxically into the RMS. Twenty-eight days later, mice were injected with low titer SAD-GFP RV (Wickersham et al., 2007b) into the core of the OB and killed after 7 days for mapping studies. Next, CRH–/–; ROSA26R-TOMA/RTVA pups were electroporated with an expression construct for rhabies-G and TVA as previously described (Arenkel et al., 2011). Twenty-eight days postelectroporation, mice were injected with SAD-GFP RV as described above. See also Supplemental Experimental Procedures.

Immunohistochemistry and Imaging

OB tissues were processed for immunohistochemistry as previously described (Fuentes et al., 2013). Briefly, free-floating sections were stained with the following primary antibodies: guinea pig anti-Parvalbumin, and rabbit anti-CRH (kindly provided by Nick Justice), followed by washing and incubation with species-specific Alexa-633 secondary antibodies. Confocal image analysis was performed using Leica TCS SPE confocal microscope. See also Supplemental Experimental Procedures.

CRH–/– and CRHR1–/– Adult-Born Neuron Proliferation, Survival, and Apoptosis

Age-matched adult (6–8 weeks old) male mice were injected with BrdU. For SVZ-based proliferation studies, animals were killed 24 hr later as and 50-µm-thick sections were taken throughout the SVZ and stained with mouse anti-BrdU and rabbit anti-Ki67 to assess proliferating cells. To assay granule cell survival, mice were killed 30 days later and processed for immunohistochemistry using mouse anti-BrdU antibody. Images were taken in the middle and outer granule cell layer, avoiding the RMS and IPL/MCL regions, and total cell numbers were counted through serial sections. See also Supplemental Experimental Procedures.

Constitutively Active CRHR1 Overexpression

A constitutively active CRHR1–EGFP fusion construct (Nielsen et al., 2000) was electroporated into the SVZ of P2 pups for morphology analysis using Neurolucida software. The construct was further subcloned into a conditional flexed adeno-associated-viral vector (Atasoy et al., 2009) and packaged into viral particles. Virus was stereotaxically injected into the RMS of CRHR1–Cre mice, and OB tissue was harvested 14 days postinjection. See also Supplemental Experimental Procedures.

Electrophysiology

Animals (P21–P35) were deeply anesthetized using isofurane, and perfused intracardially with ice-cold ACSF. Coronal olfactory bulb slices (300 µm) were placed in a room-temperature chamber mounted on an Olympus upright microscope (BX50WI), and perfused with oxygenated ACSF. Cells were depicted using fluorescence and differential interference contrast imaging. Synaptic currents were recorded using cesium-methanesulfonate based internal solutions. See also Supplemental Experimental Procedures.

In Vivo Photostimulation

Fiberoptics were generated and implanted directly over the olfactory bulb as previously described (Ung and Arenkel, 2012). Animals were allowed to recover from the surgery for 3 days prior to photostimulation. Photostimulation was performed for 3 hr using a blue laser source (CrystaLaser) controlled by a Master-8 (A.M.P.I.). See also Supplemental Experimental Procedures.

Statistical Methods

Unless otherwise indicated, statistical comparisons between experimental groups were made using Student’s t test and all error bars represent SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online http://dx.doi.org/10.1016/j.devcel.2014.07.001.

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