In the Piriform Cortex, the Primary Impetus for Information Encoding through Synaptic Plasticity Is Provided by Descending Rather than Ascending Olfactory Inputs

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Abstract

Information encoding by means of persistent changes in synaptic strength supports long-term information storage and memory in structures such as the hippocampus. In the piriform cortex (PC), that engages in the processing of associative memory, only short-term synaptic plasticity has been described to date, both in vitro and in anesthetized rodents in vivo. Whether the PC maintains changes in synaptic strength for longer periods of time is unknown: Such a property would indicate that it can serve as a repository for long-term memories. Here, we report that in freely behaving animals, frequency-dependent synaptic plasticity does not occur in the anterior PC (aPC) following patterned stimulation of the olfactory bulb (OB). Naris closure changed action potential properties of aPC neurons and enabled expression of long-term potentiation (LTP) by OB stimulation, indicating that an intrinsic ability to express synaptic plasticity is present. Odor discrimination and categorization in the aPC is supported by descending inputs from the orbitofrontal cortex (OFC). Here, OFC stimulation resulted in LTP (>4 h), suggesting that this structure plays an important role in promoting information encoding through synaptic plasticity in the aPC. These persistent changes in synaptic strength are likely to comprise a means through which long-term memories are encoded and/or retained in the PC.

Key words: long-term potentiation, naris closure, olfactory bulb, olfactory cortex, orbitofrontal cortex

Introduction

Synaptic plasticity is a cellular process, involving information storage, that is likely to enable memory of different durations. Persistent forms of synaptic plasticity, in the forms of long-term potentiation (LTP) and long-term depression (LTD), are expressed in memory processing structures such as the hippocampus (Bliss and Lomo 1973; Dudek and Bear 1992; Manahan-Vaughan 1997), as well as in structures involved in sensory information processing, such as the visual cortex (Tsanov and Manahan-Vaughan, 2007a, 2007b).

At the level of the primary visual cortex, synaptic plasticity is expressed in conjunction with the active processing of visual information by adult rodents (Tsanov and Manahan-Vaughan 2007a, 2007b). This preprocessing of visual information by the
visual cortex changes excitability levels in the hippocampus (Tsanov and Manahan-Vaughan 2009) and may thus, support hippocampal encoding of this sensory modality into a more complex representation. It is, as yet, unclear whether other primary sensory cortices share this property. Storage of perceptual information at the level of the primary sensory cortices is very likely, however. For example, in the somatosensory cortex, tactile stimulation leads to reorganization processes in humans that are believed to depend on the induction of synaptic plasticity (Hoffken et al. 2007). Furthermore, transcranial magnetic stimulation, which emulates synaptic plasticity-inducing protocols, also triggers cortical plasticity (Tegenthoff et al. 2005; Ragert et al. 2008).

More than 40 years ago, the pioneering computational neuroscientist, David Marr proposed that on the basis of their anatomical circuitry, the hippocampus, cerebellum, and piriform cortex (PC) are all ideally suited for the encoding and long-term retention of experiences (Marr 1971). Whereas, a multitude of studies have described long-term synaptic plasticity in vivo in both the hippocampus and cerebellum, very few studies have indicated that the PC exhibits long-lasting forms of plasticity. Nonetheless, the primary olfactory (piriform) cortex is likely to engage in the encoding of associative experiences. It has been proposed to engage in pattern separation and completion (Marr 1971; Haberly 2001), as well as in perceptual learning (Barnes et al. 2008; Chapuis and Wilson 2011; Shahkawat et al. 2015). The PC also engages in odor discrimination and odor rule learning (Roman et al. 1987; Chaillan et al. 1996; Saar et al. 1998; Cohen et al. 2008, 2014) indicating that it not only preprocesses olfactory information, but that it may also store information of this kind. However, although short-term changes in synaptic weights have been reported in the ascending pathway from the olfactory bulb (OB) to the PC in vitro and in anesthetized animals (Kanter and Haberly 1990; Poo and Isaacson 2007; Cohen et al. 2008), little is known as to whether more persistent (>4 h) forms of synaptic plasticity are expressed that could in turn support long-term information storage in the PC. In addition, it has been reported that one specific high-frequency stimulation (HFS) protocol has no effect on synaptic strength in the anterior PC (aPC) in vivo (Stripling et al. 1988, 1991). A key question in this regard is the source of instruction: Can patterned stimulation of the OB as the primary ascending input to the PC result in long-term synaptic plasticity, or is the contribution of top-down inputs from higher order structures required? In vitro studies suggest that associational fiber inputs may facilitate the induction of synaptic plasticity (Jung et al. 1990; Kanter and Haberly 1990). Here, in freely behaving adult rats, we compared the extent to which OB stimulation can result in synaptic plasticity in the aPC with changes elicited by orbitofrontal cortex (OFC) stimulation. We focused on the OFC because it is strongly interconnected with the olfactory cortex and is involved in odor discrimination and categorization (Illig 2005; Stalnaker et al. 2014; Cohen et al. 2015).

We tested a broad spectrum of stimulation protocols that are known to elicit synaptic plasticity of differing magnitudes, forms, and durations in the hippocampus in vivo. We report that under normal behavioral conditions, patterned stimulation of the OB does not induce synaptic plasticity in the aPC. Prolonged naris closure served to change action potential firing properties, without altering the expression of N-methyl-D-aspartate receptor (NMDAR) subunits and demonstrated that under these circumstances synaptic plasticity could be induced by OB stimulation. Strikingly, patterned stimulation of the OFC resulted in LTP of the aPC, suggesting that the OFC may control synaptic weights in this structure. Thus, we show on the one hand that the OFC plays a decisive role in the determination as to whether information is stored for longer periods in aPC synapses. On the other hand, we demonstrate that long-term synaptic information storage occurs in the PC, indicating that it may serve as a repository for olfactory memories.

Materials and Methods
Subjects

The present study was carried out in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) for care of laboratory animals. All experiments were performed according to the guidelines of the German Animal Protection Law and were approved by the North Rhine-Westphalia State Authority (Bezirksamt, Arnsberg). All efforts were made to reduce the number of animals used. Male Wistar rats were housed individually and maintained on a 12 h light/12 h dark cycle. They had ad libitum access to water and food.

Surgery

Male Wistar rats (7–8 weeks old, Charles River, Sulzfeld, Germany) underwent chronic implantation of a monopolar recording and bipolar stimulation electrode (diameter: 0.1 mm, polyurethane-coated stainless steel wire, Biomedical Instruments, Züllnitz, Germany). For examination of the ascending pathway to the PC, a stimulation electrode was implanted into the OB and a recording electrode was implanted into the dentritic layer I of the aPC (see Supplementary Fig. 1A,C). To investigate the monosynaptic component of the evoked response, the field potential component that appeared immediately after the stimulus artifact was analyzed. Coordinates for stimulation electrode: 7.9 mm anterior to bregma (AP), 1.1–1.3 mm lateral (LAT) from midline, and 1.6–2.0 mm ventral from pial surface (DV), for recording electrode: 3.2–3.7 mm AP, 3.0–3.3 mm LAT, and 5.5–7.0 mm DV based on coordinates described by Cohen et al. (2008).

In another cohort of animals, the descending pathway from the OFC was examined (see Supplementary Fig. 1B). A stimulation electrode was implanted into the OFC (3.0 mm AP, 2.0 mm LAT, and 3.8–4.2 mm DV) and a recording electrode was positioned in layer II of the aPC (3.2 mm AP, 3.3 mm LAT, and 5.4–6.0 mm DV) at a position where the evoked potential shows a negative deflection immediately after the stimulus artifact, reflecting the monosynaptic connection between OFC and aPC. The coordinates were based on those used by Cohen et al. (2008).

The electrode assembly was sealed and fixed to the skull with dental acrylic (Paladur®, Heraeus Kulzer GmbH, Hanau, Germany). Pre- and postsurgery analgesia was implemented using Meloxicam (Metacam®, Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany). Pre- and postsurgical analgesia was implemented using Meloxicam (Metacam®, Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany). Pre- and postsurgical analgesia was implemented using Meloxicam (Metacam®, Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany).

Seven to ten days after surgery recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained in the aPC by stimulating the OB, or the OFC. Throughout experiments, animals could move freely within the recording chamber (40 cm × 40 cm × 50 cm), as the implanted electrodes were connected via a flexible cable and a swivel connector to the stimulation unit (World Precision Instruments, Sarasota, FL, USA) and amplifier (A-M Systems, Sequim, WA, USA). Aside from the insertion of the connector cable at the start of the experiment, disturbance of the animals was kept to an absolute minimum.
Measurement of Evoked Potentials

fEPSPs in the aPC were generated by stimulating the OB or the OFC at a low frequency (0.017 Hz) with single biphasic square wave pulses of 0.2 ms duration per half wave. For each time-point measured during the experiments, 5 recordings of evoked responses recorded every 60 s were averaged. The first 6 time-points recorded at 5 min intervals were used as a baseline reference and subsequently obtained data points were calculated as a percentage of the mean of these 6 time-points. The fEPSP was measured as the maximum slope from the onset of the fEPSP to the trough of the first negative deflection of the evoked response. By means of an input/output curve determination (evaluation of 9 stimulation intensities from 100 μA to 900 μA in 100 μA steps), the maximum fEPSP response was identified, and for the subsequent experiment a stimulus intensity that produced 40–50% of this maximum was used to evoke fEPSP responses. After 60 min of recording, the period between samples of evoked potentials was extended to 15 min. Patterned stimulation of the OB or OFC was applied after 30 min of test-pulse recordings to investigate if persistent synaptic plasticity can be induced. Evoked responses were followed for 4 h after application of patterned afferent stimulation. Twenty-four hours later a further 1 h recording was conducted. Only animals that exhibited stable test-pulse stimulation responses for 4.5 h and the 1 h on the following day were used for experiments with application of patterned stimulation. In experiments where patterned afferent stimulation failed to induce synaptic plasticity recordings were stopped 4 h after application of the stimulation protocol.

The following stimulation protocols were used: Low-frequency stimulation (LFS) at 0.5 Hz, 1 Hz, 2 Hz, and 3 Hz were applied as 900 consecutive pulses at a stimulation intensity of 40–50% or 70% of the maximum evoked response during the input/output curve. Stimulations at 15 Hz, 25 Hz, and 50 Hz were applied as 400 consecutive pulses. HFS at 100 Hz was applied as 4 bursts of each 100 pulses with an interburst interval of 5 min. HFS at 200 Hz and 400 Hz was applied as 10 bursts of 15 pulses at either 200 Hz or 400 Hz, with an interburst interval of 10 s. Theta-burst stimulation (TBS) was applied as 3 trains with an interval of 1 min and each train consisted of 10 bursts with each 10 pulses at 100 Hz with an interburst interval of 200 ms (Cohen et al. 2008). A second TBS protocol consisted of 2 trains of each 5 bursts with a 30 s intertrain interval and an interburst interval of 200 ms. The 4 pulses of each burst were applied at 100 Hz (Staubli and Scafidi 1999).

For the ascending pathway from the OB, all protocols were tested to examine if the induction of persistent synaptic plasticity depends on a specific frequency (Table 1).

Animals with naris plugs were tested once a week with patterned afferent stimulation starting from 2 weeks after insertion of the naris plug. Stimulation protocols were applied in the following order: TBS with 3 trains, HFS at 100 Hz, LFS at 1 Hz 900 pulses at 70% intensity and 50 Hz.

To examine the descending pathway from the OFC, specific protocols were chosen: LFS at 1 Hz 900 pulses at 70% intensity, and HFS at 100 Hz and 200 Hz.

The intracortical electroencephalogram was monitored throughout in vivo experiments. None of the protocols used elicited epileptiform or seizure activity in the rats.

Postmortem Verification of Electrode Position

At the end of the study, brains were removed for histological verification of electrode localization and stained in 0.1% cresyl violet following the procedure that was described before (Hansen and Manahan-Vaughan 2015). Photomicrographs (see Supplementary Fig. 1) were taken with a digital video camera system (Visitron Systems, Puchheim, Germany) on a microscope (Leica Mikrosysteme Vertrieb GmbH). Animals with incorrectly implanted electrodes were excluded from further analysis.

Sensory Deprivation

Animals for electrophysiological experiments with sensory deprivation were implanted with chronic electrodes in the OB and aPC as described above. They were tested for stable test-pulse stimulation responses and animals exhibiting unstable recordings of test-pulse stimulation were excluded. Nasir plugs were prepared as reported by Cummings et al. (1997). One naris plug, made of silicone tubing (diameter 2.5 mm, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), surgical silk (metric 4), and filaments of unwaxed dental floss, was inserted under anesthesia (Nembutal, 52 mg/kg, i.p.) and analgesia (Meloxicam, 0.2 mg/kg, s.c.). In one cohort (N = 10), a naris plug was inserted into the nostril ipsilateral to the electrode position. A second cohort (N = 8), that underwent insertion of a naris plug into the nostril contralateral to the electrode position, served as a control. During the period after naris plug insertion, weight, appearance, behavior, and well-being of the animals were regularly checked. Two weeks after naris plug insertion, electrophysiological experiments using patterned afferent stimulation of the OB were commenced. At the end of in vivo electrophysiological recordings, animals were used for in vitro patch clamp recordings.

To examine the effect of sensory deprivation on protein expression in the aPC and OB, an additional cohort of male Wistar rats (12 weeks) underwent naris plug insertion into the right nostril (ipsilateral to aPC recordings). The plug was left in place for 3 weeks. The contralateral hemisphere served as a control.

Patch Clamp Recordings

For preparation of acute brain slices, rats were anaesthetized with isoflurane, decapitated and the brain was extracted.
rapidly in ice cold, and oxygenated sucrose cutting solution (in mM: 87 NaCl, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 1.25 Na₃H₂PO₄·H₂O, 2 d-glucose, 75 sucrose, 25 NaHCO₃). Coronal brain slices (350μm thick) containing the aPC were cut using a vibratome (VT1000S, Leica Biosystems Nussloch GmbH, Nussloch, Germany) and slices were transferred into tempered (35°C), oxygenated sucrose cutting solution and incubated for 30 min. For patch clamp recordings, slices were transferred into a recording chamber positioned on the fixed stage of a microscope (BX51WI, OLYMPUS EUROPA SE & CO. KG, Hamburg, Germany). Using infrared light, cell bodies of neurons in layer 2/3 of the aPC were visually identified and subsequently used for whole-cell patch clamp recordings. Borosilicate glass recording pipettes were filled with an intracellular solution (in mM: 97.5 potassium gluconate, 32.5 KCl, 5 EGTA, 10 HEPES, 1 MgCl₂, 4 Na₂ATP, pH 7.3, 290 mOsm). Recordings were performed in a current-clamp mode using an amplifier (EPC10 USB, HEKA Electronic Dr. Schulze GmbH, Lambrecht/Pfalz, Germany) and raw data were digitized at 10 kHz. PATCHMASTER acquisition software and AP feature software (MATLAB code developed in Department of Psychology, University of Connecticut, by Prof. M. Volgushev) were used to analyze intrinsic properties of the membrane. The resting potential was determined from the mean of 10's baseline recording. The input resistance was calculated from the slope of the linear fit of the relationship between the change in membrane potential (ΔV) and the amplitude of the applied current (duration: 600 ms, between -40 pA and +20 pA). The current necessary to evoke an action potential from the resting potential was determined as threshold current. The onset of the action potential was defined by a minimum rate of rise (set as 20 V/s). The time required to reach the half-width point of the action potential was measured from the onset of the action potential. The firing frequency properties were examined by applying square current pulses (duration 1 s) from 0 pA to 400 pA in steps of 50 pA and the firing frequency was analyzed as the number of spikes elicited during the application of each current step. Patched cells were filled with biocytin (1 mg/ml, Sigma-Aldrich, St. Louis, USA) and detected by streptavidin Cy3 (1:1000, Dianova, Hamburg, Germany). The neuronal marker NeuN (1:100, clone A60, Merck Millipore, Darmstadt, Deutschland) detected by goat anti-mouse Cy2 (1:250, Dianova) was used to differentiate between neurons and interneurons (Fig. 3F). All cells were divided into pyramidal cells, semilunar cells, and interneurons depending on patch clamp recordings and morphological properties (Haberly 1983; Suzuki and Bekkers 2007, 2011). Only pyramidal cells were used for further analysis.

**Immunohistochemistry**

A cohort of 5 animals with a unilateral naris plug was deeply anesthetized with Nembutal (52 mg/kg, i.p.) and perfused transcardially with cold Ringer’s solution containing heparin (0.2%, Roche, Basel, Switzerland) followed by 4% paraformaldehyde (PFA). The brains were stored in 4% PFA for 24 h followed by cryoprotection in 30% sucrose at 4°C for several days. The ipsilateral and contralateral hemispheres of each brain were separated and serial coronal section (30 μm) containing the aPC was prepared on a freezing microtome.

Immunohistochemistry was performed on free-floating sections as previously described (Yousef et al. 2004; Gruter et al. 2015). Briefly, after H₂O₂ pretreatment and blocking in PBS-Tx containing avidin (Avidin–biotin blocking kit, Vector Laboratories, Burlingame, CA, USA), sections were incubated with goat polyclonal anti-NMDAρ2 primary antibody (1:200, sc-1469, Santa Cruz Biotechnology, Dallas, TX, USA) in biotin (Avidin–biotin blocking kit, Vector Laboratories), overnight at room temperature (RT). Then, sections were incubated in biotinylated horse anti-goat secondary antibody (1:500, # BA 9500, Vector Laboratories), before applying avidin–biotin complex (1:1000, # PK-6100, Vector Laboratories).

For the GluN2A receptor, the biotinylated tyramine method (Adams 1992) was used. After pretreatment and blocking, sections were incubated for 5 days at 4°C in the primary antibody solution containing rabbit polyclonal anti-NMDAρ1 (1:250, #sc-9056, Santa Cruz Biotechnology). Then sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:500, #BA-1000, Vector Laboratories). Avidin–biotin complex (1:1000) was applied, before and after incubation in the amplification solution with 1% biotinylated tyramine.

Staining was visualized by a 0.05% 3,3’-diaminobenzidine solution (DAB, Sigma-Aldrich). Finally, sections were mounted onto 4% potassium chrome alum-gelatin slides, dehydrated and cover-slipped with Permount. Images of stained sections were acquired with a digital camera (MBF Europe B.V., Delft, the Netherlands) on a light microscope with Neurolucida software (MBF Europe B.V.) at a magnification of 2.5x.

Routine scrutinization of the PC using a light microscope, during immunohistochemical assessments, revealed no evidence of apoptosis as a result of naris closure (not shown).

**Western Blotting**

For western blotting analysis, a cohort of 10 animals with naris plugs was anesthetized with isoflurane, decapitated and brains were dissected. From each animal, the ipsilateral and contralateral aPC and OB were dissected and separately frozen.

Western blotting was performed as previously described (Gruter et al. 2015; Novkovic et al. 2015). Briefly, the tissue was homogenized and centrifuged (20,000 g, Eppendorf Centrifuge 5417R). Pellet and supernatant were diluted in Tris–HCl buffer containing protease inhibitor (Roche). The total protein concentration for each sample was determined using the Bradford protein assay (Ultrospec 3000, Pharmacia Biotech, Piscataway, NJ, USA). Protein samples (at least 10 μg protein per sample) were separated in sodium dodecyl sulfate (SDS) polyacrylamide gels. Electrophoresis was performed for ca. 1.5 h at 400 V and 15 mA. Then gels and polyvinyl difluoride membranes were placed into the transfer chamber (400 V, 300 mA, and 30 min). Membranes were blocked (0.1% TWEEN 20 in TBS with 5% nonfat dry milk) and afterwards, incubated overnight at 4°C in SignalBoost solution (Calbiochem) containing the primary antibody: rabbit polyclonal anti-tyrosine hydroxylase (1:2000, # AB152, Merck Millipore), rabbit polyclonal anti-GluN2A (1:1000, # 07-632, Merck Millipore), or rabbit polyclonal anti-GluN2B (1:1000, # 06-600, Merck Millipore). As a loading control, mouse monoclonal anti-β-actin (1:5000–1:500,000, # A2228, Sigma–Aldrich) was labeled. Then membranes were incubated with the secondary antibodies: anti-mouse or anti-rabbit horseradish peroxidase linked lgG (1:20,000, # NA931V, # NA932V, GE Healthcare). For every target protein, 2–4 gels (in a randomized order of samples) were performed. Protein bands were visualized using an enhanced chemiluminescence reagent (1:1, #RP2232V1, #RP2232V2, GE Healthcare) with CCD camera (Fusion Solo S, Vilber Lourmat) using VisionCapt software (v16.12, Vilber Lourmat).

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In Situ Hybridization

Animals that were used for subsequent analysis of Arc mRNA expression in the aPC were implanted with a stimulation electrode in the OFC and a recording electrode in the aPC, as described above. On the day of experiment, animals were habituated to the recording box for ca. 1 h before they were stimulated in the OFC with one burst of 100 pulses 100 Hz at a stimulation intensity of 400 μA. Five minutes after HFS, animals were sacrificed, brains were removed, shock-frozen in isopentane at −80°C on dry ice and stored at −80°C until sectioning into 20 μm thick coronal slices on a Cryostat (Leica CM3050S). Sections included the aPC and OFC (ca. +5.0 – 20.0 mm from Bregma), were mounted directly on glass slides and stored at −80°C. Sections of animals with incorrect electrode placement in the OFC were excluded from further analysis.

Compartment analysis of temporal activity by fluorescence in situ hybridization was conducted using a modified procedure used by Guzowski and Worley (2001) as described previously by our lab (Gruter et al. 2015). Arc cDNA plasmids (Entelechon GmbH, Bad Abbach, Germany) with the sequence of Lyford et al. (1995) were linearized and the antisense RNA probe labeled with digoxigenin was created (Ambion MaxiScript Kit, Invitrogen, Carlsbad, CA, USA). Yield and integrity were verified by gel electrophoresis. Per animal, one glass slide (with at least 3 sections containing the aPC) was left at RT for 1 h. Slides were fixed in 4% PFA, washed in 2x saline-sodium citrate buffer (SSC) and placed in acetic anhydride solution. After washing in 2x SSC they were left in 2x SSC. Slides were placed in a humid chamber (1:1, 2x SSC, and 50% deionized formamide solution) and prehybridization buffer (Sigma-Aldrich) was added. Digoxigenin-labeled DNA probes (1 ng/μl) in hybridization buffer (Sigma-Aldrich) were applied on the slides and hybridization in a humid chamber at 56°C lasted ca. 17 h. Then, slides were placed in 56°C 2x SSC (3 × 5 min, each) and afterwards in 2x SSC containing RNase (1 μg/ml, Sigma-Aldrich). Before final rinsing in TBS (3 × 5 min, each), they were placed for 10 min at 37°C in 2x SSC, 10 min at 56°C in 0.5x SSC, 30 min at 56°C in 0.5x SSC, 10 min at RT in 0.5x SSC, and 2 times for 5 min at RT in 1x SSC.

For signal detection, slices were pretreated with 3% H2O2 in 1x SSC for 15 min and incubated for 70 min in 0.2% TBS-Tween 20 containing 20% avidin (Avidin–biotin blocking kit, Vector Laboratories) and 1% bovine serum-albumin (BSA). Arc-digoxigenin was detected by anti-digoxigenin-POD Fab fragment (1:400, #11207 733910, Roche) in TBS-Tween containing 1% BSA and 20% biotin (Avidin–biotin blocking kit, Vector Laboratories) for 90 min. Signal was enhanced using biotinylated tyramine (Adams 1992) in TBS for 20 min. The Arc signal was visualized by streptavidin Cy5 (1:2000, Dianova) and nuclei by 4’,6-diamidino-2-phenylindole (1:10 000, DAPI, Invitrogen) in 1% BSA TBS-Tween for 90 min. After rinsing and air drying, slides were mounted (SCR-38447, Dianova).

Arc mRNA expression within the nuclei of neurons in lower layer 2 of the aPC was examined. Therefore, z-stacks were obtained using a Zeiss Apotome at 63× magnification. For each animal, z-stacks of both hemispheres of 3 sections were obtained. The hemisphere contralateral to the electrode placement was used as control. Z-stacks were chosen to contain representative regions of aPC lower layer 2.

Data Analysis and Statistics

Statistical analysis was conducted using Statistica software (Version 12, StatSoft, Inc., USA).

Electrophysiology in Freely Behaving Animals

All fEPSP responses recorded during in vivo electrophysiological experiments were expressed as a mean percentage ± standard error of the mean (SEM) of the average baseline value and visualized using GraphPad Prism software (GraphPad Software Inc., USA). To analyze differences in responses to patterned afferent stimulation, between groups, an analysis of variance (ANOVA) with repeated measures was conducted. For every stimulation protocol, the effect of patterned stimulation was compared with test-pulse stimulation (Figs 1A,B and 4A–C and Table 1). For experiments with naris plug animals, the effect of each stimulation protocol was compared between the 2 cohorts (Fig. 2A–D).

Patch Clamp

For statistical comparisons of neuronal characteristics (e.g., resting membrane potential) between both (open and closed naris) groups, unpaired Student’s t-tests were used (Fig. 3D; see Supplementary Fig. 4). For comparison of firing frequency properties of action potentials, ANOVA with repeated measures was performed (Fig. 3G).

Immunohistochemistry

The optical density of immunohistochemically stained sections was calculated using the “Color deconvolution” plugin (Ruifrok et al. 2003) and conversion from red, green, blue (RGB) to 8-bit format in ImageJ (1.51d, Wayne Rasband, National Institutes of Health, USA). For each section, the background staining was subtracted from the regions of interest (lower layer 1 of aPC), using the anterior part of the anterior commissure. To scale the independent plates and staining sessions, a generalized residual sum of squares algorithm in R software was used (Kreutz et al. 2007). Statistical analysis was performed to compare protein levels the ipsilateral (closed) with the contralateral (open) side using paired Student’s t-tests in Statistica software (Fig. 3A,B; see Supplementary Fig. 3). Results were expressed as signal intensity (a.u.) ± SEM and visualized using GraphPad.

Western Blotting

For quantification of western blotting, the volume of the protein band and the corresponding actin band were determined for each sample using VisionCapt software. Blots with missing or blurred actin bands were excluded from the analysis. For each sample of each blot, the ratio of the volume of the protein to the volume of actin was calculated. After scaling in R software (residual sum of squares algorithm), the results were visualized as signal intensity (a.u.) ± SEM using GraphPad and statistical analysis (paired t-test) was performed in Statistica software (Fig. 2E, see Supplementary Fig. 2).

In Situ Hybridization

To examine Arc mRNA expression after HFS in the OFC, complete nuclei were marked in each z-stack using ImageJ software and nuclei were checked for Arc mRNA expression. The percentage of Arc mRNA positive neurons of all neurons was counted for each z-stack during an experimenter-blind analysis. For each animal, the mean of 3 z-stacks taken per ipsilateral and contralateral hemisphere was calculated. Results were visualized using GraphPad and unpaired t-test was performed to examine differences between hemispheres (Fig. 4E).
Results

Patterned Stimulation of the OB of Freely Behaving Rats Fails to Elicit Synaptic Plasticity in the aPC

Adult male rats (7–8 weeks old) underwent implantation of a stimulating electrode in the OB and a recording electrode in the aPC, to enable subsequent recordings of evoked responses from freely behaving animals (see Supplementary Fig. 1). We first applied LFS at 1 Hz in the OB, a frequency that elicits robust LTD (Manahan-Vaughan 1997). This protocol failed to result in any changes in synaptic strength in the aPC, however (test-pulse stimulation versus 1 Hz LFS: $F_{1,42} = 0.55, P = 0.463, N = 22$, ANOVA, Fig. 1A). HFS at 100 Hz, that elicits robust LTP in the hippocampus (Manahan-Vaughan 1997), also failed to elicit changes in synaptic strength (test-pulse stimulation versus 100 Hz HFS: $F_{1,40} = 0.6, P = 0.441, N = 21$, ANOVA, Fig. 1B). To clarify if this absence of effect was specifically related to the stimulation frequencies or protocols used, we examined the effects of OB stimulation at frequencies ranging between 0.5 Hz and 400 Hz, as well as using theta-burst stimulation (TBS). None of these protocols resulted in synaptic plasticity (Table 1).

Naris Closure Reveals Frequency-Dependent Synaptic Plasticity in the aPC

Given reports in the literature that the aPC expresses synaptic plasticity in vitro (Jung et al. 1990; Kanter and Haberly 1990; Franks and Isaacson 2005; Poo and Isaacson 2007), we wondered whether the absence of effects in the freely behaving rat reflected a resistance, rather than an inability, to express plasticity under the conditions tested. Postnatal olfactory deprivation results in enhanced synaptic potentiation in the aPC (Franks and Isaacson 2005). We therefore assessed whether naris closure (Cummings et al. 1997) changes the insensitivity of the aPC to patterned stimulation of the OB. Following 2 weeks of unilateral naris closure, TBS of the OB resulted in LTP.
that lasted for more than 4 h in the aPC. This effect was only apparent ipsilaterally (N = 10) to naris closure when compared with naris closure on the contralateral side (N = 8) (F(1, 16) = 13.604, P < 0.01, ANOVA, Fig. 2A). HFS at 100 Hz resulted in a tendency toward an increase of evoked potential magnitude in the aPC ipsilaterally, compared with responses evoked contralaterally to naris closure (F(1, 18) = 4.029, P = 0.062, ANOVA, Fig. 2B). Here, responses were significantly greater after HFS compared with test-pulse stimulated controls (F(1, 18) = 11.232, P < 0.01, N = 10, ANOVA, not shown). By contrast, stimulation at 1 Hz or 50 Hz (ipsilateral: N = 9, contralateral: N = 8) failed to elicit changes in synaptic strength in the aPC (t(9) = 0.09, P = 0.448, N = 5 each, t-test, see Supplementary Fig. 2). Similarly, NMDAR subunit expression was equivalent in layer 1 of the aPC that was contralateral to naris closure (t(9) = 0.84, P = 0.412, N = 5 each, t-test, Fig. 3A–C). Layer 2, that others have reported as exhibiting changes in GluN2B mRNA after olfactory deprivation in “mice” (Kim et al. 2006), also exhibited no differences (GluN2B: t(9) = 0.385, P = 0.72, GluN2B: t(9) = −0.204, P = 0.848, N = 5 each, t-test, see Supplementary Fig. 2). Western blot analysis revealed no change in subunit protein levels (GluN2A: Δt(9) = 0.385, P = 0.72, GluN2B: Δt(9) = −0.204, P = 0.848, N = 5 each, t-test, see Supplementary Fig. 2). Similarly, NMDAR subunit expression was equivalent in layer 1 of the aPC that was ipsilateral (“closed”) or contralateral (“open”) to naris closure (GluN2A: t(10) = −0.421, P = 0.695, GluN2B: t(9) = 0.916, P = 0.412, N = 5 each, t-test, Fig. 3A–C). Layer 2, that others have reported as exhibiting changes in GluN2B mRNA after olfactory deprivation in “mice” (Kim et al. 2006), also exhibited no differences (GluN2B: t(9) = −0.84, P = 0.448, N = 5 each, t-test, see Supplementary Fig. 3). Although passive membrane properties of aPC neurons remained unchanged (see Supplementary Fig. 4), we detected changes in firing properties of pyramidal cells of the aPC (Fig. 3D–H) when cells that were ipsilateral (N = 11, n = 18) or contralateral (N = 11, n = 14) to naris closure were compared. Here, in particular, an increase in the duration of the action potential was evident in terms of the half-width of the potential (t(14) = 2.543, P < 0.05, t-test, Fig. 3D). The firing frequency remained unchanged between hemispheres (F(1, 30) = 0.014, P = 0.91, ANOVA, Fig. 3G,H). These findings suggest that reorganization at the level of the NMDAR does not occur, but naris closure may change neuronal sensitivity to depolarization.

The OFC Supports the Induction of Synaptic Plasticity in the aPC

Our observation, that the PC resists expressing synaptic plasticity following OB stimulation, while retaining an intrinsic ability to express LTP after olfactory sensory deprivation provoked the question as to the circumstances under which the aPC might express synaptic plasticity in the absence of a manipulation such as naris closure. The OFC is involved in odor categorization and is strongly interconnected to the olfactory cortex, including the PC (Illig 2005; Stalnaker et al. 2014). Thus, we explored whether patterned stimulation of the OFC might induce synaptic plasticity in the aPC.

We observed that HFS of the OFC at 100 Hz resulted in LTP in the aPC compared with nonstimulated rats (F(1,12) = 26.649, P < 0.001, N = 7, Fig. 4A), whereas LFS at 1 Hz did not change synaptic strength (F(1,12) = 3.426, P = 0.089, N = 7, ANOVA, Fig. 4B). Increasing the stimulation intensity to 200 Hz did not result in an improvement of LTP (F(1,14) = 0.582, P = 0.458, N = 8, ANOVA, Fig. 4C) suggesting that the regulation of LTP in the aPC by the OFC is constrained to a narrow frequency range. Examination of Arc mRNA expression in the aPC, as a marker for activity-dependent neuronal activity (Guzowski et al. 1999), revealed an increase in Arc mRNA expression in the aPC ipsilateral to HFS of the OFC compared with the contralateral aPC (t(14) = 2.372, P < 0.05, N = 6 each, t-test, Fig. 4E–G). This supports that stimulation of the OFC has a direct impact on neuronal activity in the aPC.
stimulation of the OFC, which is involved in odor perception and categorization (Li et al. 2010; Stalnaker et al. 2014), resulted in LTP (>4 h), but not LTD in the aPC (Fig. 4). We propose that it is the top-down regulation by the OFC that provides an important “impetus” to the PC for information encoding in the form of synaptic plasticity. It also shows that the aPC is capable of long-term maintenance of changes in synaptic strength.

This study is the first to examine whether persistent synaptic plasticity, that is typically elicited by afferent patterned stimulation in a broad range of frequencies (in structures such as the hippocampus), occurs in the ascending olfactory pathway to the aPC in freely behaving rodents. In accordance with in vivo studies that focused on attempts to induce LTP following one application of one specific afferent frequency (Racine et al. 1983; Strippling et al. 1988, 1991), none of the afferent stimulation protocols were effective in generating any long-lasting changes in synaptic weight in the monosynaptic response, although the protocols are highly effective in generating synaptic plasticity that lasts for days in the hippocampus in vivo (Manahan-Vaughan 1997, 1998, 2000). Nonetheless, synaptic plasticity in the PC has been reported following activation of the ascending fibers in studies performed in vitro (Jung et al. 1990; Kanter and Haberly 1990; Franks and Isaacson 2005; Poo and Isaacson 2007) and in anesthetized animals (Cohen et al. 2008), but these effects were small and difficult to induce (Jung et al. 1990; Kanter and Haberly 1990; Cohen et al. 2008). Furthermore, in vitro effects seem to be linked to an early postnatal period (Franks and Isaacson 2005; Poo and Isaacson 2007). Altogether, our data obtained from adult behaving rats suggest that information transfer from the ascending pathway alone is not sufficient to promote long-term information encoding via synaptic plasticity in the aPC.

It was quite striking that none of the large range of stimulation protocols that we tested were effective in eliciting synaptic plasticity in the aPC (Fig. 1 and Table 1), especially given the importance of persistent synaptic plasticity for long-term information storage in ostensibly comparable structures such as the hippocampus (Manahan-Vaughan and Brauneewell 1999; Whitlock et al. 2006; Nabavi et al. 2014). Furthermore, other primary sensory cortices, such as the visual cortex, express synaptic plasticity in conjunction with visual information processing (Tsanov and Manahan-Vaughan 2007b, 2007a). One possibility is that, despite the ostensibly similar input and “wiring” of the hippocampus and PC (Marr 1971; Haberly 2001), the “hippocampus-derived” afferent stimulation patterns in no way emulated intrinsic patterns that can be expected to originate from the OB. Notwithstanding this, stimulation patterns in the range of breathing (1–3 Hz; Welker 1964; Walker et al. 1997) or sniffing-related OB oscillations (TBS, 15 Hz, 25 Hz, 50 Hz; Chapman et al. 1998; Ravel et al. 2003; Kay et al. 2009) were also ineffective in triggering synaptic plasticity. These discrepancies can possibly be explained by the distinctive neuroanatomy of the olfactory system compared with the other sensory systems. For example, in contrast to other primary sensory cortices, sensory stimuli, in the form of odor information, do not undergo initial thalamic processing, with projections originating from the olfactory epithelium, continuing from the OB to the paleocortical olfactory cortex in a broadly distributed and nontopographic manner (Illig and Haberly 2003; Sosulski et al. 2011). This contrasts with the topographical manner in which visual, auditory, and somatosensory information is processed: projecting from the periphery via thalamic nuclei to neurons of the neocortical primary sensory cortices (Kaas 1997). The nontopographical projections of the olfactory system could mean, in turn, that the

Discussion

In this study, we explored whether persistent forms of synaptic plasticity are expressed in the aPC following stimulation of ascending or descending afferent pathways to this structure. Strikingly, despite testing a broad range of frequencies to stimulate the OB, we could not elicit either LTP or LTD in the aPC of freely behaving adult rats under standard physiological and behavioral conditions (Fig. 1 and Table 1). This does not reflect an absence of an intrinsic ability of this structure to express synaptic plasticity, however: Naris closure, that is known to change aPC sensitivity (Best and Wilson 2003; Franks and Isaacson 2005) facilitated the expression of LTP following OB stimulation (Fig. 2). This raised the question as to which physiological conditions could lead to synaptic plasticity in the aPC, in the absence of naris closure. We observed that patterned
populations of synapses that were activated by afferent stimulation of the OB in our study possessed a degree of heterogeneity that precluded them from expressing monosynaptic plasticity (Sosula et al. 2011). Another consideration is that a precise modulation of the intrinsic circuitry of the PC and the associated feedforward and feedback inhibition within this circuitry (Suzuki and Bekkers 2012) is required for sensory information processing at the level of synaptic plasticity.

Although other in vivo studies in awake rodents also found an absence of long-lasting synaptic plasticity after HFS in the monosynaptic component of the evoked potential (Racine et al. 1983; Stripling et al. 1988, 1991), one of these studies reported kindling induced plasticity after daily HFS in vivo (Racine et al. 1983). Kindling protocols are used to induce epilepsy in rodents (Loscher 2002; Chauvette et al. 2016) and this procedure is known to cause significant changes in the circuitry of the PC (Loscher and Ebert 1996). The protocols we used in our study were much milder than the kindling protocol used by the studies mentioned above, and at no time did we detect seizure activity in our rats.

Given reports that synaptic plasticity can be induced in the PC in vitro and in anesthetized rats (Jung et al. 1990; Kanter and Haberly 1990; Franks and Isaacson 2005; Poo and Isaacson 2007; Cohen et al. 2008), we wondered whether the success of these studies related to a relative suppression of intrinsic excitability that was related, in turn, to the absence of olfactory inputs (in the cortical slice) and reduced excitatory tonic (in the anesthetized rodent). Another possibility is that in adulthood the PC loses a readiness for the expression of synaptic plasticity that is characteristic of neonatal cortical circuitry (Cair and Malenka 1995; Kirkwood et al. 1995; Best and Wilson 2003; Franks and Isaacson 2005; Poo and Isaacson 2007). A third consideration is that sensory deprivation triggers increases in sensitivity to sensory inputs and emulates neonatal plasticity propensity in sensory structures such as the visual and somatosensory cortex (He et al. 2006; Chung et al. 2017).

This motivated us to examine whether synaptic plasticity can be induced in the aPC after prolonged naris closure. We found that under these circumstances the aPC expresses LTP in response to TBS of the OB (Fig. 2). Similar effects have been demonstrated in an in vitro study of early developmental plasticity in the aPC, where an enhanced response of the aPC to weak TBS, and reduced excitability upon stimulation of the ascending fibers have been reported following naris closure in neonatal rats (Best and Wilson 2003; Franks and Isaacson 2005). In addition, changes in morphology of the OB and aPC have been reported after unilateral olfactory deprivation (Meisami 1976; Cummings et al. 1997; Wilson et al. 2000), and changes in intrabulbar projections are triggered that are believed to restore the OB projections to a state equivalent to that achieved in early development (Marks et al. 2006; Cummings and Belluscio 2010).

Although permanent unilateral naris closure can result in apoptosis of neurons in the OB and PC (Heimer and Kalil 1978; Friedman and Price 1986; Frazier and Brinjes 1988; Leung and Wilson 2003; Cummings and Belluscio 2010), in the present study, temporary naris closure did not alter the gross anatomy of the aPC. Others have reported that naris cautery in adult rats triggers weak apoptosis in the PC that peaks at around 5 days and returns to control levels 10–20 days after closure (Leung and Wilson 2003). Our assessments took place at least 2 weeks after temporary naris closure, and thus, we can assume that apoptosis played a negligible role, if any, in the synaptic plasticity effects we detected.

In the present study, manifestation of LTP in the aPC, in response to OB stimulation after naris closure, was not associated with changes in NMDAR subunit expression (Fig. 3A, B; see also Supplementary Figs 2 and 3), in contrast to reports from a study that examined changes in GluN2B mRNA in the aPC of “mice” under similar circumstances (Kim et al. 2006). This suggests that changes on the mRNA level may not impact on the protein level of these receptor subunits, or that the mouse effects are species-specific. The absence of alterations in passive neuronal properties, such as the resting membrane potential found in our study (see Supplementary Fig. 4), suggests that generalized changes in excitability did not occur. However, the change in action potential width that we observed (Fig. 3D) indicates that more specific alterations in the sensitivity of neurons to afferent stimulation were triggered by naris closure. The alterations induced by naris closure may include a change in the sensitivity or expression of A-type K+ channels: Blocking these channels promotes synaptic potentiation in the ascending fibers of the aPC in vitro (Johenning et al. 2009). A modification of this kind would result in a more efficient transfer of ascending olfactory information from distal apical dendrites to aPC neurons, which under normal circumstances is not very reliable (Bathellier et al. 2009). Finally, under condition of sensory deprivation, the induction of synaptic plasticity is enhanced, whereas under normal circumstances the ascending pathway does not enable the induction of synaptic plasticity.

Another consideration here is that through naris closure we emulated a situation whereby no olfactory information was processed by the recipient PC. Even in the absence of directed sniffing, or confrontation with a specific odor, breathing can be expected to provide an ongoing “stream” of olfactory information, that in turn, may result in “background noise” against which the PC must identify salient olfactory information that should possibly be stored in the form of associative memory. Naris closure will have served to remove this kind of background noise, thereby improving signal-to-noise ratios during our attempts to induce LTP through OB stimulation. This interpretation offers an explanation as to why synaptic plasticity can be elicited in slice preparations of the PC (Jung et al. 1990; Kanter and Haberly 1990; Franks and Isaacson 2005; Poo and Isaacson 2007) and in anesthetized animals (Cohen et al. 2008): In the former case, olfaction is completely absent, and in the latter case, general anesthesia will have reduced global excitability in the PC thus, provided a less noisy backdrop upon which LTP-inducing protocols are likely to be more effective.

An absence of ability of lateral olfactory tract-aPC synapses to “autonomously” trigger and/or express synaptic plasticity may actually be advantageous to olfactory information processing. The physiology of the OB speaks against a role for this structure in triggering synaptic plasticity: Rapid odor desensitization and adaptation processes occur at the sensor level (Getchell and Shepherd 1978; Zufall and Leinders-Zufall 2000) suggesting that action potential firing may not be sustained long enough to trigger synaptic plasticity. Furthermore, it is unclear how the OB would “know” that a particular odor is salient enough to require encoding and storage in the PC. Sniffing behavior induces strong oscillatory coupling between OB and aPC and may thereby increase the probability of signal transmission to the aPC (Litaudon et al. 2008). Beside triggering changes in intrinsic oscillations in the olfactory system by sniffing (Chapman et al. 1998; Martin et al. 2006), changes in oscillatory activity are also triggered in associated structures such as OFC and amygdala (Chapuis et al. 2009). These changes in turn may make the aPC more receptive to the induction of
Synaptic plasticity. Finally, if the odor is behaviorally relevant, higher order structures may then “instruct” retention of an odor category/odor item by facilitating the induction of synaptic plasticity in this structure.

The question thus arises as to which circumstances can generate enough input signal resolution such that synaptic plasticity can occur in the aPC in the absence of naris closure. Here, as a high-order input structure, the OFC is a likely candidate: The OFC is strongly interconnected to the PC (Illig 2005) and is believed to engage in conscious odor perception and in odor categorization (Li et al. 2010; Stalnaker et al. 2014). In the present study, we observed that OFC stimulation resulted in LTP in the aPC. As the PC may engage in odor perception, as well as pattern completion and separation (Barnes et al. 2008; Chapuis and Wilson 2011; Shakhawat et al. 2014), the PC is likely to “remember” its past experiences. Considering the properties of the OFC it is possible that this region assists the PC in achieving odor discrimination and categorization. This top–down regulation of information processing in the aPC puts the OFC in an ideal position to either modulate, or even dictate when, synaptic plasticity may be engaged in the aPC.

The ability of the OFC to directly impact on information storage via synaptic plasticity in the aPC was confirmed by examining intranuclear expression of the immediate early gene, Arc, as a result of HFS of the OFC. Somatic Arc expression reflects experience-dependent information encoding at the neuronal level (Guzowski et al. 1999). In our study, neuronal Arc expression increased significantly in the ipsilateral aPC following LTP induction via OFC stimulation, in line with the triggering of long-term information storage in the aPC. We detected no changes in expression in the contralateral cortex, however. The PC of both hemispheres communicates with each other via commissural projections (Haberly and Price 1978; Luskin and Price 1983) but commissural communication between aPCs has rarely been explored. However, single unit responses can be categorized depending on nonresponding and responding units to unilateral (ipsi- or contralateral) or bilateral odor stimulation (Wilson 1997) suggesting that not every odor stimulation results in a bilateral response. This suggests in turn that the intensity of electrophysiological stimulation may be decisive in recruiting activation of the contralateral aPC. In the hippocampus of behaving rats stimulation of the Schaffer collateral/commissural pathway of one hemisphere evoked a response in the contralateral CA1 region that was typically smaller in magnitude than the ipsilaterally evoked field potential (Kemp and Manahan-Vaughan 2012). The fact that field potentials evoked by commissural pathway stimulation are smaller in the contralateral hemisphere (at least for the hippocampus), combined with the lower level of Arc induced by OFC stimulation in the ipsilateral aPC, suggest that the lack of contralateral expression of Arc in the aPC relates to the lower level of synaptic responses elicited.

Although our study supports an important role for the OFC in enabling aPC plasticity, it may not comprise the "sole" instigator of aPC plasticity. Our analysis focused on the components of the field potential that emerged immediately after the stimulus artifact and thus should correspond to a monosynaptic input. We cannot, however, entirely exclude that associational inputs (triggered by OFC stimulation) may have contributed to this component of the field potential. Daily HFS of the OB results in potentiation that is selective to late component of the evoked potential (Stripling et al. 1988, 1991). This suggests that the associative circuitry of the olfactory cortex also contributes to the expression of synaptic potentiation in vivo. Further evidence for a contribution of associative inputs to PC plasticity derives from sensory deprivation studies in neonatal rats that showed that naris closure for 30 days starting on postnatal day 1 had no effect on lateral olfactory tract-evoked responses in the aPC, but enhanced responses elicited by stimulation of associational connections (Best and Wilson 2003). These effects may be specifically related to an early stage of postnatal development, however, as in the present study, OB–aPC responses were enhanced by sensory deprivation.

Finally, our findings suggest a contribution of the OFC to aPC plasticity, but also do not rule out the possibility that other regions such as the posterior PC, the lateral entorhinal cortex, parts of amygdala, or the mediodorsal thalamus or the intrinsic circuitry of the aPC, which play a role in odor perception (Courtiol and Wilson 2017), may have properties that similarly support synaptic plasticity within the aPC.

In conclusion, our data confirm that in freely behaving adult rats, synaptic information storage in the form of persistent synaptic plasticity in the aPC does not typically occur following sole, patterned stimulation of the ascending olfactory pathway, despite the fact that the aPC possesses an intrinsic ability to express LTP. Under circumstances where the OFC is activated, prolonged synaptic plasticity, in the form of LTP occurs, however, in the aPC. This top–down instruction of the aPC by the OFC seems quite intuitive given the known involvement of the PC in odor discrimination and odor rule learning (Roman et al. 1987; Chaillan et al. 1996; Saar et al. 1998; Cohen et al. 2008, 2015), along with the role of the OFC in supporting odor discrimination and categorization. This finding also suggests that top–down control of olfactory information processing is a major determinant of long-term information storage in the aPC, and that the aPC may serve as a repository of memories with regard to odor discrimination and categorization.

Authors’ Contributions
D.M.-V. devised the concept and experimental strategy of the study. Experiments were conducted by C.S. and D. M-V. Data analysis and interpretation were conducted by both authors. D.M.-V. wrote the article together with C.S.

Supplementary Material
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