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# **Chronic facial inflammatory pain-induced anxiety is associated with bilateral deactivation of the rostral anterior cingulate cortex**

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## **Abstract**

Patients with chronic pain, especially orofacial pain, often suffer from affective disorders, including anxiety. Previous studies largely focused on the role of the caudal anterior cingulate cortex (cACC) in affective responses to pain, long-term potentiation (LTP) in cACC being thought to mediate the interaction between anxiety and chronic pain. But recent evidence indicates that the rostral ACC (rACC), too, is implicated in processing affective pain. However, whether such processing is associated with neuronal and/or synaptic plasticity is still unknown. We addressed this issue in a chronic facial inflammatory pain model (complete Freund's adjuvant model) in rats, by combining behavior, Fos protein immunohistochemistry and *ex vivo* intracellular recordings in rACC slices prepared from these animals. Facial mechanical allodynia occurs immediately after CFA injection, peaks at post-injection day 3 and progressively recovers until post-injection days 10-11, whereas anxiety is delayed, being present at post-injection day 10, when sensory hypersensitivity is relieved, but, notably, not at post-injection day 3. Fos expression reveals that neuronal activity follows a bi-phasic time course in bilateral rACC: first enhanced at post-injection day 3, it gets strongly depressed at post-injection day 10. *Ex vivo* recordings from lamina V pyramidal neurons, the rACC projecting neurons, show that both their intrinsic excitability and excitatory synaptic inputs have undergone long-term depression (LTD) at post-injection day 10. Thus chronic pain processing is associated with dynamic changes in rACC activity: first enhanced and subsequently decreased, at the time of anxiety-like behavior. Chronic pain-induced anxiety might thus result from a rACC deactivation-cACC hyperactivation interplay.

**Key words (not included in the title):** trigeminal pain, complete Freund's adjuvant (CFA), Fos immunohistochemistry, whole-cell patch-clamp recordings, long-term depression (LTD)

## 1. Introduction

Chronic pain is a widespread and highly debilitating condition caused by tissue or nerve injury, referred to as inflammatory and neuropathic pain, respectively. Whatever its origin, chronic pain in humans is associated with cognitive deficits and emotional changes, including anxiety, fear and depression (Muñoz and Esteve, 2005; Miller and Cano, 2009; Wiech and Tracey, 2009). Consistently, pain-induced anxiodepressive-like behavior can be observed in neuropathic (Narita et al., 2006; Suzuki et al., 2007; Roeska et al., 2008; Leite-Almeida et al., 2009; Seminowicz et al., 2009; Sellmeijer et al., 2018) as well as inflammatory rodents (Parent et al., 2012).

Functional imaging studies in humans suggest that the anterior cingulate cortex (ACC) is involved in both pain (Treede et al., 1999; Apkarian et al., 2005; Moisset and Bouhassira, 2007) and anxiety processing (Tan et al., 2017). However, it is now clear that ACC does not represent a single functional entity. As distinct cingulate subdivisions – ACC and mid and posterior cingulate cortices – differently contribute to pain processing (Vogt, 2005; Vogt, 2016; Tan et al., 2017), sub-regions within the ACC itself – namely the rostral ACC (rACC), corresponding to perigenual areas 24b, portions of perigenual 24a and caudodorsal area 32, and caudal ACC (cACC), including portions of postgenual areas 24a and 24b (Vogt and Peters, 1981; Vogt and Paxinos, 2014) – need to be considered when deciphering ACC circuits that influence pain processing. Most animal studies addressing the cellular mechanisms of ACC-mediated pain processing have targeted the cACC. There, neuronal firing is facilitated (Ning et al., 2013; Blom et al., 2014; Santello and Nevian, 2015; Shen et al., 2015) and excitatory synaptic transmission strengthened (Wu et al., 2005; Zhao et al., 2006; Xu et al., 2008; Li et al., 2010; Bie et al., 2011; Ning et al., 2013; Chen et al., 2014; Koga et al., 2015; Shen et al., 2015; Chen et al., 2016) under chronic pain conditions. Importantly, disruption of such cACC plasticity alleviates both sensory hypersensitivity (Wei et al., 2001; Wu et al., 2005; Zhao et al., 2006;

Xu et al., 2008; Li et al., 2010; Koga et al., 2015; Shen et al., 2015; Chen et al., 2016) – sensory thresholds actually increase – and anxiety-like behavior (Koga et al., 2015; Sellmeijer et al., 2018). This is consistent with the hypothesis that potentiated neuronal activity within the cACC contributes to sensory hypersensitivity – the sensory component of pain – and behavioral anxiety (Bliss et al., 2016). Accordingly, directly activating cACC produces sensory hypersensitivity (Calejesan et al., 2000; Tang et al., 2005; Kang et al., 2015).

The rACC appears to specifically process the affective component of pain. Lesion, chemical inactivation or astrocytic inactivation of the rACC alleviate pain-induced aversion, but not sensory hypersensitivity, in the setting of both inflammatory (Johansen et al., 2001; Chen et al., 2013) and neuropathic pain (LaGraize et al., 2007; Qu et al., 2011; Bannister et al., 2017; Juarez-Salinas et al., 2019). However, whether pain-induced anxiety is associated with neuronal and/or synaptic plasticity within the rACC is unknown. Surprisingly, both direct activation of rACC neurons in the absence of a peripheral noxious stimulus (Johansen and Fields, 2004) and inhibition of subcortically projecting layer 5 rACC pyramidal neurons in neuropathic mice (Meda et al., 2019) appear to be aversive.

In the present study, we aimed at characterizing the neuronal and/or synaptic changes in rACC that are associated with pain processing and its anxiety-like consequence. We used a facial model of inflammatory pain – subcutaneous injection of Complete Freund's adjuvant (CFA) into the rat upper lip (Alba-Delgado et al., 2018) – since, in humans, fear ratings are significantly higher for trigeminal than for extra-cephalic pain (Schmidt et al., 2016). To assess rACC neuronal changes, we both measured the expression of an anatomical markers of neuronal activity, Fos protein and recorded from layer V pyramidal neurons, the rACC projecting neurons (whole-cell patch-clamp recordings in *ex vivo* slices). Because pain experience and pain-induced anxiety exhibit different time courses (see for example Sellmeijer et al., 2018),

we assessed the anxiety-like behavior and neuronal properties of rACC neurons at two time points after the initiation of chronic facial inflammatory pain.

## **2. Material and methods**

### *2.1. Animals*

Male Sprague-Dawley rats (Charles River, France), weighing about 150 g at their arrival, were housed 3–4 per cage at 22°C under 12 h light/dark cycles (lights on 8 P.M.) with *ad libitum* access to food and water. All efforts were made to minimise the number of animals used. All experimental procedures followed the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the Directive 2010/63/UE of the European Parliament and of the Council and French Decree 2013–118 on the protection of animals used for scientific purposes. Protocols used in this study were approved by the local animal experimentation committee: CEMEAA “Comité d’Ethique en Matière d’Expérimentation Animale Auvergne” (#CE 28-12) and the French Ministry for Research.

### *2.2. Inflammatory model of facial pain*

Rats were quickly anaesthetised (for 4-5 min) using a mask with 5% halothane and injected with 20 µL of either NaCl 0.9% (control rats) or Complex Freund’s Adjuvant (CFA rats) subcutaneously between the 1<sup>st</sup> and the 2<sup>nd</sup> line of vibrissae at the left side. CFA was obtained by diluting 100 mg of dried *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) in: Tween 80 (1.66 mL) paraffin oil (10 mL), NaCl 0.9% (6.66 mL). CFA was stored at 4°C until use and kept 30 min at room T°C before injection. The success of CFA injection was confirmed by the development of an oedema (diameter: 0.8 to 1.2 cm) immediately after injection.

### *2.3. Behavioral tests*

Behavioral testing was performed during the dark phase, between 8:00 A.M. and 3:00 P.M. in a quiet room under low light conditions. While each rat went through repeated von Frey tests, Elevated plus maze or Light-dark box were performed on independent sets of animals at each time point (post-injection days 3 and 10) (Fig. 1).

### *2.3.1. Facial mechanical sensitivity.*

The protocols used for behavioral sensory testing are similar to those described previously (Boyer et al., 2014). Rats were first acclimated to the glass chamber (0.6 x 0.6 m square) for behavioral testing and to innocuous mechanical stimulation during 4 days before CFA/saline injection using randomized series of von Frey (VF) filaments (1.0–6 g; BIOSEB, Vitrolles, France), applied onto a region between the left vibrissa pad and the upper left lip, with carefully avoiding touching any vibrissae. Mechanical sensitivity of the skin surrounding the site of tissue damage (secondary hyperalgesia) was then assessed every day for 3 or 11 days after CFA or saline injection. Cephalic withdrawal thresholds were determined by applying VF filaments (maximum filament strength: 10 g) using the descending-ascending method (Chaplan et al., 1994). Each filament was applied to the skin 5 times for 5 seconds. VF filament threshold in gram was equal to the filament evoking a withdrawal response of the head 3 times out of 5 trials. The evaluating experimenter was blind to the experimental group.

### *2.3.2. Anxiety-like behavior.*

Rats were assessed for anxiety-like behavior using either the Elevated Plus Maze or the Light-Dark Box at Days 2-3 or 10-11 after CFA or saline injection (Fig. 1).

*2.3.2.1. Elevated plus maze (EPM) (Noldus, Wageningen, The Nederland).* EPM includes two open (OA) and two closed (CA) arms (width: 10 cm, length: 50 cm, wall height for closed arms: 40 cm) elevated from 50 cm from the ground, crossing at a squared-shape central platform (10 x 10 cm). On test day, rats were first habituated for 25 min to the testing room and then placed into the EPM, in front of the OA, opposite to the experimenter. After 5 s, animal behavior was

recorded for 5 min using a CCD camera (Panasonic CCTV). The total travelled distance, times spent in OAs and CAs and number of entries into OAs were automatically measured using EthoVision XD-9 (Noldus).

2.3.2.2. *Light-dark box (LDB) (EJM, Cournon, France)*. The LDB apparatus consists of two compartments (35 x 35 x 35 cm each): a white, brightly illuminated compartment (ambient light; around 100 lux) and a dark one (black box, around 10 lux) with a central door (7.5 x 7.5 cm) allowing rats to go from one to the other (transition). On test day, rats were placed in the lit compartment, their head opposite to the door. Rats were allowed to move freely between the two compartments with door open for 5 min. After 5 s, the distance travelled in each box, total number of transitions, time spent in each compartment and latency to enter the dark one were automatically measured using the same software as for the EPM.

#### 2.4. *Fos protein immunohistochemistry*

Thirty min after the EPM test (Fig. 1), rats were deeply anaesthetized [intraperitoneal (i.p.) urethane:  $> 1.5 \text{ g.kg}^{-1}$  body weight] and transcardially perfused with 100 mL of warm (37°C) heparinized saline (25 IU heparin.mL<sup>-1</sup>) followed by cold (10°C) 500 mL of 4% paraformaldehyde, 0.03% picric acid in 0.1 M phosphate-buffered saline, pH 7.6 for 15 min. Brains were removed, and cryoprotected overnight in a sucrose (30%) azide (5%) solution at 4°C. Coronal sections were cut on a freezing microtome (40 µm thick) and collected in a 0.05 M Tris-buffered saline (TBS).

After coronal sections had free-floated in 1% normal horse serum for 1 h, immunohistochemistry reaction was carried out with a polyclonal rabbit antibody against Fos protein (1:3000, Santa Cruz, Reference SC-52, Dallas, TX, USA; overnight at room T°C) using biotinylated horse anti-rabbit followed by avidin-biotin-peroxidase complex (30 min at room temperature; ImmPRESS, Abcys, Vector Laboratories, Burlingame, CA, USA). Fos



immunoreactivity was visualized in sections using 3,30-diaminobenzidine tetrahydrochloride (DAB) and ammonium nickel sulfate (kit Vector Peroxidase substrate DAB). All immunolabels were diluted in 0.25% bovine serum albumin, 0.3% Triton X-100 in TBS, and in all cases, sections were rinsed in TBS several times before and after each incubation. Finally, sections were rinsed in TBS and transferred to gelatinized slides before being coverslipped with DPX (Sigma-Aldrich, St. Louis, MO, USA). Neither omission of the primary antibody nor incubation of sections in inappropriate secondary antibodies provides specific staining.

The rACC was delineated upon the Paxinos and Watson atlas (Paxinos and Watson, 2007). Counts were performed on 7 levels (Bregma + 3.7 mm to + 1.7 mm) by an observer who was blind to the experimental group. Computer assisted bright field images of representative labeling were obtained with a CCD color video camera (Sony DXC-950P) connected to a Nikon Optiphot-2 microscope for DAB labeled slides. Images were exported to ImageJ (v 1.46r) to automatically counting neurons.

## *2.5. Ex vivo electrophysiological recordings*

### *2.5.1. Slice preparation.*

After the LDB test (Fig. 1), rats were deeply anesthetized with chloral hydrate (7%, i.p) and decapitated. Brains were quickly removed and immersed into ice-cold sucrose-based low- $\text{Ca}^{2+}$  and high- $\text{Mg}^{2+}$  artificial cerebrospinal fluid (aCSF) containing (in mM): sucrose (230), KCl (2.6),  $\text{CaCl}_2$  (0.5),  $\text{MgCl}_2$  (7),  $\text{NaH}_2\text{PO}_4$  (1.2),  $\text{NaHCO}_3$  (25) and glucose (25). Coronal slices (350- $\mu\text{m}$ -thick) containing the rACC (3.7 to 2.7 mm anterior to Bregma; Paxinos and Watson, 2007) were prepared using a Leica VT1200S Vibratome (Leica Microsystems, Wetzlar, Germany). Slices were then incubated at 30°C for at least 1 h and then allowed to rest at room temperature (20-23°C) in aCSF containing (in mM) NaCl (130), KCl (2.6),  $\text{CaCl}_2$  (1.2),  $\text{MgCl}_2$  (2.5),  $\text{NaH}_2\text{PO}_4$  (1.2),  $\text{NaHCO}_3$  (25) and glucose (11). A single slice was

transferred to a recording chamber and submerged in aCSF containing (in mM) NaCl (130), KCl (2.6), CaCl<sub>2</sub> (2.5), MgCl<sub>2</sub> (1.25), NaH<sub>2</sub>PO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (25) and glucose (11) at room T°C superfusing the slice at  $\approx 3 \text{ mL}\cdot\text{min}^{-1}$ . All cutting, holding and recording external solutions were saturated with 5% CO<sub>2</sub>/95% O<sub>2</sub> (pH 7.4; 310–320 mOsm).

### *2.5.2. Electrophysiological patch-clamp recordings.*

Whole-cell patch-clamp recordings were obtained from visually identified layer V pyramidal neurones within the rACC using infrared DIC-IR videomicroscopy (upright microscope: Zeiss Axioskop2 FS plus, Germany; CCD video camera: Hamamatsu, Japan). Electrodes (5 to 7 M $\Omega$ ) were made from thin-wall borosilicate capillaries (outer diameter: 1.5 mm, inner diameter: 1.12 mm, WPI, Sarasota, FL, USA) using a horizontal puller (Model P-97, Sutter Instruments, Navato, CA, USA) and filled with an internal solution containing (in mM) K gluconate (128), NaCl (20), ethylene glycol-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (EGTA) (1), HEPES (10), CaCl<sub>2</sub> (0.3), MgCl<sub>2</sub> (1), Na<sub>2</sub>ATP (2) and Na<sub>2</sub>GTP (0.3). For data acquisition, recordings were low-pass filtered at 10 kHz with a Bessel filter and sampled at 1 kHz using double patch clamp EPS 10 amplifier (HEKA, Germany) and Patchmaster acquisition software (HEKA, Germany).

### *2.5.3. Analysis.*

Resting membrane potential (RPM) was measured as the mean potential in current-clamp mode for the first seconds after break-in. During the course of the recordings, input ( $R_i$ ) and series resistances ( $R_s$ ) were periodically monitored using a 500-ms-long, 10-pA-hyperpolarizing current pulse. Cells were included in the analysis if they met the following criteria: RPM  $< -60 \text{ mV}$ , and  $R_s < 40 \text{ M}\Omega$ . To evaluate intrinsic excitability, neurons were injected with depolarizing current pulses (500-ms-long, ranging from 50 to 350 pA, with 10 pA increments) in current-clamp mode from about  $V_m = -70 \text{ mV}$  at which the neuron was kept (holding potential). Curves of the number of action potential firing as a function of injected

current were then constructed. To assess rheobase, the minimal depolarizing current injected at the soma able to generate an action potential, 3 consecutive current pulses were delivered at each current intensity. A ramp of current (from 0 pA to 2 times the rheobase, in 1 s) was used to assess action potential voltage threshold: it was the voltage at which the value of  $dV/dt$  exceeded 50 mV/ms. All the above analyses were performed using Clampfit 10 (Molecular Devices, Sunnyvale, CA, USA).

Excitatory postsynaptic currents (EPSC), recorded in voltage-clamp mode from layer V pyramidal neurons, were evoked by 0.1-ms current pulses, using a monopolar glass stimulating electrode (filled with normal aCSF, 2-3 Mohm) placed in layer 2-3 of rACC, in the presence of a blocker of fast GABA<sub>A</sub> receptor-mediated inhibitory transmission (picrotoxin 50  $\mu$ M) and glycine (1  $\mu$ M). Paired pulse ratio (ratio of the amplitude of the second EPSC to that of the first one or PPR) was evaluated by delivering pairs of identical stimuli with interpulse intervals ranging from 35 to 300 ms. Stimulus pairs were delivered five times at each interpulse interval. Evoked-EPSCs were analysed using Fitmaster (HEKA, Germany). To measure the AMPA/NMDA ratio, after measuring evoked EPSC amplitude at -70 mV, CNQX was added and evoked EPSC amplitude was measured at +40 mV.

## *2.6. Drugs*

Bicuculline and glycine were purchased from Sigma-Aldrich and CNQX from Tocris.

## *2.7. Statistical analysis*

All the data are presented as mean  $\pm$  SEM. In each group, N is for the number of tested rats and n, for the number of recorded neurons. GraphPad Prism (v8.4.0, GraphPad Inc., La Jolla, CA, USA) was used for statistical analysis. Two-way repeated (RM) or not repeated measures analysis of variance (ANOVA) was used to compare more than two groups followed

by the Tukey HSD, Newmann-Keuls or Fischer (for comparison between groups) or the Dunnett's multiple comparison post-tests (for comparison with baseline values). *F*-values were expressed with their associated degrees of freedom (DFn and DFd) and their corresponding *p*-value. Factors were designed as followed: *Drug*, for comparisons between vehicle and CFA; *Time*, for comparisons between different time points; *Group*, for comparisons between vehicle and CFA-injected rats and ipsi and contralateral sides; *Current*, for comparisons between different current intensities; and *Interaction*, for effect between two factors on the dependent variable.

### 3. Results

#### 3.1. Facial CFA injection induces a facial mechanical allodynia and a delayed anxiety

To investigate the role of rACC in pain-induced sensory hypersensitivity and anxiety-like behavior, we used the facial CFA model of chronic inflammatory pain in rats (Alba-Delgado et al., 2018). Mechanical sensitivity of the skin surrounding the site of tissue inflammation, within the area of the secondary hyperalgesia, was assessed using von Frey filaments. CFA injection led to a profound decrease in the thresholds for evoking facial withdrawal to mechanical stimuli on not only the ipsilateral but also the contralateral side of the face (two-way ANOVA,  $F_{\text{group}(3,481)} = 97.9, P < 0.0001$ ;  $F_{\text{time}(11,481)} = 14.7, P < 0.0001$ ;  $F_{\text{Group*Time}(33,481)} = 5.7, P < 0.001$ ; Fig. 2). Nevertheless, noxious mechanical thresholds of the face were predominantly lowered on the injection side ( $P < 0.01$  and  $P < 0.001$  ipsi vs. contra from post-injection days 1 to 7 by Tukey's post-test following two-way ANOVA; Fig. 2). Facial mechanical allodynia was observed from post-injection days 1 to 8 and had completely recovered by post-injection day 9 when compared to saline-treated rats ( $P > 0.05$  by Tukey's post-test) and to baseline ( $P > 0.05$  by Dunnett's post-test). This indicates that facial CFA injection into the left vibrissa pad produced a predominantly ipsilateral, long-lasting mechanical allodynia. Notably, there was no

difference in the mechanical thresholds measured in the two groups of CFA-treated rats and sacrificed at post-injection days 3 and 10 (mechanical thresholds at baseline:  $9.4 \pm 0.20$  g and  $9.4 \pm 0.20$  g and at post-injection day 3:  $2.3 \pm 0.6$  g and  $2.7 \pm 0.54$  g, in rats sacrificed at post-injection days 10 and 3, respectively;  $N = 18$  in the two groups; Unpaired t-test:  $P > 0.05$ ).

Since chronic pain also induces anxiety in addition to sensory hypersensitivity (Wu et al., 2017; Sellmeijer et al., 2018), we also assessed the anxiety-like behavior at two time points after CFA injection: at post-injection day 3, when mechanical hypersensitivity is maximum, and at post-injection day 10, when mechanical allodynia had recovered. We used two behavioral tests, the Elevated Plus Maze (EPM) and the Light-Dark Box (LDB), in two different cohorts of rats ( $N = 24$  and  $56$  respectively). Anxiety-like behavior in EPM is usually inferred from a decrease in the time spent in the open arms (OAs) and/or a decrease in the number of OA crossings. There were significant differences in the time spent in the OAs (Fig. 3A1) and closed arms (CAs; Fig. 3A1) and in the number of OA entries (Fig. 3A2). Tukey's post-test following two-way ANOVA (see Legend to Fig. 3) revealed that the time spent in OAs ( $P < 0.01$ ) and the number of OA crossings ( $P < 0.01$ ) were both lower in CFA- vs. saline-treated rats at post-injection day 10 but not at post-injection day 3. Such effect could not be attributed to reduced locomotor activity as here there was no difference in the total travelled distance ( $P > 0.05$ ; Fig. 3A3) between the four groups. This suggests that CFA-treated rats exhibit an anxiety-like behavior only at post-injection day 10.

In LDB, reduced latency to first transition, time spent in the light compartment and number of transitions are commonly used phenotypic markers of anxiety-like behavior in rodents. Tukey's post-test following two-way ANOVA (see Legend to Fig. 3) revealed that CFA-treated rats left the light compartment faster ( $P < 0.001$ ; Fig. 3B1), spent more time within the dark compartment ( $P < 0.001$ ; Fig. 3B2) and exhibited a smaller number of transitions ( $P < 0.001$ ; Fig. 3B3) than saline-treated rats at post-injection day 10 but not at post-injection day 3.

Thus, combined results obtained with EMP and LDB indicate that the facial CFA injection produces an anxiety-like behavior, in addition to mechanical hypersensitivity. But whereas mechanical hypersensitivity develops right after facial CFA injection, anxiety-like behavior is delayed. Moreover, it is still present when mechanical hypersensitivity has recovered. These different time courses allow studying the molecular/cellular changes in rACC associated with mechanical hypersensitivity in the absence of anxiety-like behavior and *vice versa*.

### 3.2. Facial CFA injection produces a bidirectional modulation of rACC activity

To gain an appreciation of rACC functional activity associated with mechanical allodynia and anxiety-like behavior, we analysed the expression of the Fos protein, an anatomical marker of activation, within rACC at post-injection day 3 – when mechanical hypersensitivity peaks but there is no anxiety-like behavior (N = 6 in CFA- and saline-treated groups) – and post-injection day 10 – after pain relief, when anxiety-like behavior is still present (N = 6 and 4-5 in CFA- and saline-treated groups, respectively; Fig. 4). Fos immunoreactivity within rACC varied according to treatment and post-injection day (two-way ANOVA; ipsilateral rACC:  $F_{Group(3,38)} = 27.5, P < 0.001$ ;  $F_{Time(1,38)} = 147.2, P < 0.001$ ;  $F_{Group*Time(3,38)} = 74.8, P < 0.001$ ; contralateral rACC:  $F_{group(3,38)} = 20.1, P < 0.001$ ;  $F_{Time(1,38)} = 109.5, P < 0.001$ ;  $F_{Group*Time(3,38)} = 56.1, P < 0.001$ ). Tukey's post-test following two-way ANOVA showed that, at post-injection day 3, Fos immunoreactivity within rACC was strongly increased in CFA- vs. saline-treated rats ( $P < 0.001$ ; Fig. 4D). Such enhancement occurred within all layers (II-III and V) of bilateral rACCs. Surprisingly, Fos immunoreactivity was conversely strongly reduced within layers II-III of ipsilateral ( $P < 0.01$ ) and contralateral ( $P < 0.01$ ) rACCs in CFA- vs. saline-treated rats at post-injection day 10. Fos immunoreactivity tended to be also reduced in layers V of bilateral rACCs, too, although not significantly ( $P > 0.05$ ; Fig. 4D).

These results indicate that rACC neuronal activity undergoes bidirectional changes: first increasing – when mechanical hypersensitivity is present but not anxiety-like behavior – and

subsequently strongly decreasing – when mechanical hypersensitivity has recovered but anxiety-like behavior is still present. Notably, these bidirectional changes in neuronal activity occur in both superficial and deeper layers of bilateral rACCs.

### *3.3. Rostral ACC layer V pyramidal neurons exhibit depressed both intrinsic excitability and layer II-III inputs at post-injection day 10.*

We then tested whether neuronal and/or synaptic changes contribute to such bidirectional modulation of rACC neuronal activity. According to Fos expression, superficial (layer II-III) as well as deeper (layer V) neurons in bilateral rACCs exhibit elevated and, subsequently, reduced activity after facial CFA injection. When considering information flow through rACC, it goes from thalamic inputs to layer IV neurons, from layer IV to layer II-III neurons and then from layer II-III to layer V neurons. Therefore, we chose to investigate electrophysiological changes in layer V pyramidal neurons, as they are positioned downstream to layer II-III neurons and are the projecting rACC neurons. We recorded from rACC layer V pyramidal neurons in *ex vivo* slices prepared from CFA- and saline-treated rats at post-injection days 3 (N = 12 and 15, respectively) and 10 (N = 16 and 13, respectively).

Passive membrane properties of randomly sampled rACC layer V pyramidal neurons were: resting membrane potential ( $V_{mr} = -69.0 \pm 0.4$  mV; n = 92) and input resistance ( $R_i = 140 \pm 5$  Mohms; n = 92). Neither  $V_{mr}$  (two-way ANOVA:  $F_{Drug*Time(1, 88)} = 1.84, P > 0.1$ ) nor  $R_i$  (two-way ANOVA:  $F_{Drug*Time(1, 88)} = 0.50, P > 0.4$ ) were different between groups. We also asked whether CFA injection altered the intrinsic excitability of rACC layer V pyramidal neurons by examining neuronal responses to 500-ms depolarizing current steps (Fig. 5A) from a baseline potential of -70 mV. All layer V pyramidal neurons displayed typical regular spiking discharge patterns (McCormick et al., 1985). The number of evoked action potentials (APs) obviously depended on current intensity (RM two-way ANOVA,  $F_{Current(3, 264)} = 428.12, P <$

0.01) but also on treatment and post-injection day (RM two-way ANOVA;  $F_{\text{Drug*Time}(3, 264)} = 3.30, P < 0.05$ ). Fischer's post-test following two-way ANOVA revealed that, at post-injection day 10, 130 to 200 pA current injections elicited less APs in CFA- vs. saline-treated rats ( $P < 0.05$  to  $0.01$ ) whereas there was no difference between CFA- and saline-treated rats at post-injection day 3 ( $P > 0.8$ ). Thus only the F-I curve of CFA-treated rats at post-injection day 10 was shifted to the right compared with F-I curves in all other groups of rats which were otherwise superimposed (Fig. 5A). These results reveal that the intrinsic excitability of rACC layer V pyramidal neurons is depressed 10 days after CFA injection. Such decreased intrinsic excitability was not due to altered  $V_{\text{mr}}$  or  $R_{\text{i}}$  of the recorded neurons (see above) but rather to increased rheobase (two-way ANOVA;  $F_{\text{Drug*Time}(1, 88)} = 7.42, P < 0.01$ ) (Fig. 5D). Finally, the properties of individual APs recorded at rheobase – threshold, amplitude, duration, time to peak and area – were similar in the four groups of rats (Table 1).

We also assessed the effect of CFA injection on excitatory synaptic transmission onto the recorded rACC layer V pyramidal neurons ( $n = 73$  neurons). A stimulating electrode was lowered into layer II-III to activate layer II-III afferents to layer V pyramidal neurons. Evoked excitatory postsynaptic currents (eEPSCs) were recorded at a holding potential of  $-70$  mV in the presence of the GABA<sub>A</sub> receptor antagonist, picrotoxin ( $50 \mu\text{M}$ ), and glycine ( $1 \mu\text{M}$ ). Comparison of eEPSC amplitude vs. stimulus intensity relationships (input-output curves) in CFA- and saline-treated rats at post-injection days 3 and 10 showed that eEPSC amplitude depended on the treatment and post-injection day (RM two-way ANOVA;  $F_{\text{Drug*Time}(1, 69)} = 5.16, P < 0.05$ ; Fig. 5B). Neumann-Keuls' post-test following two-way ANOVA revealed that eEPSC responses to the three highest stimulus intensities were smaller ( $P < 0.05$  to  $0.01$ ) at post-injection day 10 in CFA- vs. saline-treated rats. On the other hand, eEPSC amplitudes were similar in CFA- and saline-treated animals at post-injection day 3 ( $P > 0.3$ ). Again, only the input-output curve in CFA-treated rats at post-injection day 10 was shifted to the right compared



with input-output curves in all other groups of rats which were otherwise superimposed (Fig. 5B). These results indicate that layer II-III excitatory inputs onto rACC layer V pyramidal neurons are depressed at post-injection day 10.

Chronic pain-induced anxiety has been shown to be associated with a presynaptic form of long-term potentiation (LTP) in layer II-III of the cACC (Koga et al., 2015). Therefore, we measured the paired-pulse ratio (PPR; Fig. 5C) which is commonly used as a measure of presynaptic function (Zucker and Regehr, 2002). Paired-pulses were delivered at 20- to 300-ms intervals. PPR percentage consistently decreased in all groups when the inter-pulse interval increased until reaching a plateau at longer intervals ( $\geq 100$  ms). PPR depended upon treatment and post-injection day (RM two-way ANOVA;  $F_{Drug*Time(1, 71)} = 7.28$ ,  $P < 0.01$ ). Neumann-Keuls' post-test indicated that PPRs were larger ( $P < 0.01$ ) at post-injection day 10 in CFA- vs. saline-treated rats. On the other hand, they were similar ( $P > 0.3$ ) in CFA- and saline-treated rats at post-injection day 3. Again, inter-stimulus interval-PPR relationships at post-injection day 3 in CFA- and saline-treated rats and at post-injection day 10 in saline-treated rats were superimposed (Fig. 5C). Notably, only CFA-treated rats at post-injection day 10 exhibited paired-pulse facilitation ( $\approx 115\%$ ). There was rather paired-pulse depression in other groups. PPR elevation indicates that reduced transmitter release contributes to the depression of layer II-III excitatory inputs onto rACC layer V pyramidal neurons at post-injection day 10.

Some neurons ( $n = 32$ ) were then assessed for another marker of synaptic plasticity: the AMPA/NMDA ratio, which is commonly used as a measure of postsynaptic function (Thomas et al., 2001; Ungless et al., 2001) (Fig. 5E). The AMPA/NMDA ratio depended upon treatment and post-injection day (two-way ANOVA;  $F_{Drug*Time(1, 28)} = 8.93$ ,  $P < 0.01$ ). Neumann-Keuls' post-test revealed that the AMPA/NMDA ratio, on the one hand, tended to increase at post-injection day 3 but, on the other hand, was significantly decreased ( $P < 0.01$ ) at post-injection day 10 in CFA- vs. saline-treated rats. These results indicate that, together with presynaptic

mechanisms, postsynaptic ones, similar to AMPA receptor internalization (Snyder et al., 2001) also contribute to depressing layer II-III excitatory inputs onto rACC layer V pyramidal neurons.

Altogether, these results indicate that rACC layer V pyramidal neurons undergo depression of intrinsic excitability and layer II-III excitatory inputs at post-injection day 10. Moreover, such depression of layer II-III excitatory inputs involves both pre- and post-synaptic mechanisms.

#### **4. Discussion**

We show that in a rat model of chronic facial inflammatory pain, mechanical hypersensitivity and anxiety-like behavior display different time courses: at post-injection day 3, mechanical hypersensitivity is maximum but anxiety-like behavior is not yet present whereas, at post-injection day 10, mechanical hypersensitivity has recovered but not anxiety-like behavior. Fos protein expression reveals the dynamics of neural activity in rACC: being first potentiated and, subsequently, strongly depressed, i.e. below baseline, in superficial as well as deep layers of bilateral rACC. Deactivation of rACC at post-injection day 10 involves reduced intrinsic excitability as well as excitatory transmission of/onto layer V pyramidal neurons, the major rACC output neurons.

Our behavioral analysis indicates that the anxiety-like consequence of chronic trigeminal inflammatory pain develops at least 3 days after sensory hypersensitivity and persists after it. Interestingly, anxiety-like behavior develops also within 3 days after formalin injection into the vibrissa pad in rats (Araya et al., 2020). Regarding the hind-paw CFA model of chronic inflammatory pain in rats, anxiety-like behavior occurs between 1 day and 4 weeks following CFA injection (Parent et al., 2012; Wang et al., 2015; Wu et al., 2017). On the other hand, in mice, increased (Narita et al., 2006; Mutso et al., 2012; Chen et al., 2013; Refsgaard et al., 2016;

Hofmann et al., 2017) as well as unchanged anxiety-like behaviors (Urban et al., 2011; Maciel et al., 2013; Liu et al., 2015; Sheahan et al., 2017; Pitzer et al., 2019) have been reported. Nevertheless, when the anxiety-like consequence of hind-paw inflammatory pain is present in mice, it is detected 1 day to 4 weeks after CFA injection (Narita et al., 2006; Chen et al., 2013; Refsgaard et al., 2016; Hofmann et al., 2017). Notably, neuropathic rodents also exhibit delayed anxiety-like behaviors, but weeks to months after nerve lesion (for review see Kremer et al., 2021), thus suggesting that the anxiety-like behavior develops even slower under neuropathic than under inflammatory conditions (see Discussion in Pitzer et al., 2019). Our results also indicate that the anxiety-like behavior can persist after recovery from mechanical hypersensitivity as in inflammatory rats (hind-paw CFA model) (Wu et al., 2017) and neuropathic mice (Dimitrov et al., 2014). Altogether, these results indicate that sensory hypersensitivity and anxiety-like behavior do not exhibit temporal synchrony, with the latter being delayed compared with the former.

Using anatomical markers of activity, it was shown that inflammatory pain is associated with cACC neuronal hyperactivity (Wei et al., 2001; Li et al., 2010; Thibault et al., 2014). Our analysis of Fos protein expression at post-injection day 3 demonstrates that inflammatory pain is associated with rACC neuronal hyperactivity, too. Moreover, such rACC hyperactivity, as cACC one (Chen et al., 2014), is bilateral. However, at this time point, there was no change in the intrinsic excitability and excitatory synaptic transmission of/onto rACC layer V pyramidal neurons under our inflammatory conditions. In neuropathic rodents, rACC (Meda et al., 2019) and cACC (Blom et al., 2014) layer V pyramidal neurons become hyperexcitable and their excitatory inputs, in rACC (Meda et al., 2019) and cACC (Chen et al., 2014), get stronger. It is nevertheless important to note that, in these very neuropathic mice, whereas basolateral amygdala inputs to rACC layer V projecting neurons get stronger, mediodorsal thalamic ones get weaker (Meda et al., 2019). Moreover, only inputs onto a sub-class of cACC layer V

projecting neurons, the corticospinal projecting ones, were shown to be potentiated (Chen et al., 2014). Variability might thus come due to input specificity and/or the nature, tissue or nerve, of injury. However, notably, excitatory inputs onto cACC layer II-III pyramidal neurons constantly exhibit long-term potentiation (LTP) – though various forms (see below) – after peripheral tissue damage/inflammation (Wu et al., 2005; Zhao et al., 2006; Bie et al., 2011) as well as nerve lesion (Xu et al., 2008; Li et al., 2010; Koga et al., 2015; Sellmeijer et al., 2018). But there are species-specific variabilities. Thus, under inflammatory conditions, strengthened excitatory inputs onto cACC layer II-III pyramidal neurons involve presynaptic mechanisms in mice (Zhao et al., 2006) but postsynaptic ones in rats (Bie et al., 2011). Finally, other mechanisms than intrinsic and synaptic plasticity, such as network rearrangements or altered functional connectivity, can account for changes in functional neuronal activity. Thus, stimulus-evoked firing of layer V pyramidal neurons is enhanced (i) in ACC of neuropathic mice, following down-regulation of hyperpolarization-activated cyclic nucleotide-regulated (HCN) channels in dendrites, thus facilitating the integration of excitatory inputs (Santello and Nevian, 2015), (ii) in S1 of neuropathic mice, after concomitant reductions in somatostatin-expressing and parvalbumin-expressing inhibitory neuronal activities (Cichon et al., 2017) and, (iii) in ACC of mice in which reduced expression of astrocytic  $\text{Na}^+$ ,  $\text{K}^+$ -ATPases impairs astrocytic glutamate uptake (Romanos et al., 2020). Finally, facilitated layer II-III pyramidal neuronal output can enhance layer V pyramidal neuronal activity, as facilitated S1 output enhances ACC activity under chronic inflammatory conditions (Eto et al., 2011).

At post-injection day 10, inflammatory rats exhibited a strongly reduced Fos protein expression in superficial and deeper layers of bilateral rACC, suggesting that decreased rACC activity is correlated with the anxiety-like behavior. Moreover, *ex vivo* whole-cell patch-clamp recordings revealed that depression of both intrinsic excitability and excitatory synaptic transmission of/onto layer V pyramidal neurons contribute to such rACC deactivation. It is now

well established that long-term depressions (LTD) of both intrinsic excitability and excitatory transmission can be synergistically induced following sustained afferent stimulation (Daoudal et al., 2002; Gasselin et al., 2017). Depressed intrinsic excitability and excitatory synaptic transmission of/onto rACC layer V pyramidal neurons at post-injection day 10 may thus result from the initial hyperactivity at post-injection day 3. Such a scenario would account for the delayed onset of anxiety-like behavior after peripheral tissue inflammation (see above). The prelimbic cortex (PLC) next to rACC within the medial prefrontal cortex (mPFC) is also involved in pain processing (for review see Tan and Kuner, 2021). Interestingly, the excitability (Wang et al., 2015; Zhang et al., 2015; Cheriyan et al., 2018; Mitrić et al., 2019) and excitatory synaptic inputs (Kelly et al., 2016; Metz et al., 2009) of/onto PLC layer V pyramidal neurons are significantly depressed in inflammatory as well as neuropathic rodents. Moreover, reversing PLC deactivation, by knocking down the cyclin-dependent kinase 5, alleviates hind-paw CFA-induced pain and anxiety (Wang et al., 2015). Very recently, Hogrefe *et al.* (*In press*) reported that excitatory synapses onto layer V pyramidal neurons in the mouse cACC can undergo LTD but that this LTD is impaired in neuropathic conditions. Importantly, such LTD phenotype is not correlated with mechanical hypersensitivity as it persists after the time of healing from the persistent pain, suggesting that it is rather relevant to the emotional processing of pain. Thus the various modulations of synaptic plasticity, specifically of LTD, at excitatory synapses onto layer V pyramidal neurons in the different ACC areas – setting of synaptic and intrinsic LTD in rACC and suppression of synaptic LTD in cACC – may contribute to the development of specifically the anxiety-like consequences of pain. Altogether, these results support a model in which deactivation of rACC, and more generally of mPFC, promotes the anxiety-like consequence of pain. Pain-induced anxiety would thus rely on reciprocal relationships between, on the one hand, hyperactive cACC (Koga et al., 2015; Santello and Nevian, 2015) and, on the other hand, hypoactive rACC (present results) and PLC (Wang et al., 2015) within the mPFC.

Interestingly, according to a fMRI study, the experience of sadness in humans is associated with concomitant limbic activation and pre-frontal deactivation (Mayberg et al., 1999).

Finally, since inflammatory mechanical pain hypersensitivity had also recovered at post-injection day 10, synaptic and intrinsic LTDs might thus contribute to sensory recovery but not to anxiety. This is very unlikely. First, the cellular change in cACC underlying sensory hypersensitivity is an enduring potentiation of excitatory synaptic transmission – i.e. a LTP-like phenomenon. Indeed, disruption of LTP in the cACC alleviates pain hypersensitivity (Wei et al., 2001; Wu et al., 2005; Zhao et al., 2006; Xu et al., 2008; Li et al., 2010; Koga et al., 2015; Shen et al., 2015; Chen et al., 2016). Thus chronic pain- and activity-dependent changes in excitatory synaptic transmission appear to be similar (for review see Josselyn and Tonegawa, 2020; Reyes-García and Escobar, 2021). This raises the possibility that recovery from inflammatory pain is an extinction-like process. One theory of memory extinction is engram silencing as extinction protocols also produce depotentiation; that is, the reversal of LTP (for review see Josselyn and Tonegawa, 2020; Reyes-García and Escobar, 2021). According to this theory, recovery from inflammatory pain would thus be a simple return of potentiated ACC excitatory synaptic transmission to baseline levels. Another theory posits that extinction reflects not the unlearning of the original engram but rather a new learning (Myers and Davis, 2007; Orsini and Maren, 2012). Recovery from inflammatory pain would then be a novel, extinction-like learning process overlying the original pain experience (Meulders, 2020). One possibility to reconcile these two theories is that, during recovery from inflammatory pain, within ACC are neurons undergoing depotentiation during extinction and others mediating the new learning (Orsini and Maren, 2012). Anyhow, whether recovery from inflammatory pain at post-injection day 10 is an extinction-like learning or unlearning process, it can account, at most, for a reversal of ACC potentiated excitatory synaptic transmission to baseline levels, but certainly not for true synaptic and intrinsic LTDs. Second, neither lesion (Johansen et al., 2001;

Qu et al., 2011; Juarez-Salinas et al., 2019) nor pharmacological inhibition of rACC (LaGraize et al., 2007), can interrupt the sensory discriminative aspects of pain experience; i.e. mechanical hypersensitivity persists. It has to be noted, however, that in neuropathic mice treated with direct anodal current transcranial stimulation (tDCS), long-lasting pain relief is associated with reduced Fos expression in the rACC, suggesting that the resting activity of these regions is lower than normal over the period of analgesia post-tDCS (Gan et al., 2021).

## **5. Conclusion**

In summary, provided that the organization of murine ACC circuitry mimics that in humans, these results confirm that the different parts of ACC, namely rACC and cACC, need to be considered when deciphering ACC circuits that influence pain experience. They reveal that, under inflammatory conditions, rACC neuronal activity undergoes a biphasic dynamic in conjunction with behavior: that is, not only a hyperactivation, with mechanical hypersensitivity, but also a deactivation, subsequently with anxiety-like behavior. Though purely correlational – future experiments should use pharmacology, optogenetic ... to directly manipulate rACC neuronal activity – these data strongly suggest that rACC deactivation contributes to the setting of the anxiety-like consequence of chronic pain. In such scenario, the anxiety-like consequence of chronic pain would thus result from the interplay between cACC activation and rACC/mPFC deactivation. The required regional perturbation for remission of chronic pain-induced anxiety-like consequences might then be neither deactivating cACC nor reactivating rACC but reinstating their interplay.

*Abbreviations* : AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APs, Action potentials; cACC, Caudal anterior cingulate cortex; CFA, Complete Freund's adjuvant; EPM, Elevated plus maze; EPSC, Excitatory postsynaptic current; GABA, Gamma-aminobutyric acid; LDB, Light-dark box; LTD, Long-term depression; LTP, Long-term potentiation; NMDA, N-Methyl-D-aspartate; PPR, Paired-pulse ratio; rACC, Rostral anterior cingulate cortex

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## **CRediT authorship contribution statement**

ED, FJ and AA designed the experiments. ED performed the *ex vivo* electrophysiological experiments. FJ and AD carried out the behavioral and immunostaining experiments. ED and AA wrote the original draft. AA and RD reviewed and edited the manuscript. AA and RD acquired funding. All authors discussed the manuscript. All authors read and approved the final manuscript.

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## **Declaration of Conflicts of interest**

All authors declared no financial or other conflicts of interest involved.



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## Legends to the figures

**Figure 1. Schematic representation of the experimental design.** Rats were first acclimatized to the glass chambers for behavioral testing and to innocuous mechanical stimulation with von Frey (VF) filaments during 4 days before injection. They then received artificial complete Freund's adjuvant (CFA) or saline into the left vibrissa pad on Day 0. Ipsilateral and contralateral facial mechanical thresholds (using VF filaments; grey rectangles) were assessed just before and every day after injection. At post-injection days 2-3 (referred to as post-injection day 3) and 10-11 (referred to as post-injection day 10), two separate cohort of rats were assessed for anxiety, using the Elevated Plus Maze (EPM) or Light-Dark Box (LDB) (white rectangles). Rats were then killed for Fos protein immunohistochemistry or *ex vivo* electrophysiological recordings in rACC (black rectangles).

**Figure 2: Facial mechanical thresholds (VF filaments).** Withdrawal thresholds to VF filaments applied to the ipsi- (empty and filled circles) and contralateral sides (empty and filled diamonds) of the face (with respect to injection site) were measured in rats just before and for 11 days after subcutaneous injection of CFA (empty symbols; N = 18) and saline (filled symbols; N = 17) into the left vibrissa pad. There was no difference between responses measured before and after saline injection, indicating that injection alone produced no sensitization to mechanical stimuli. On the other hand, withdrawal thresholds strongly decreased after CFA injection. Withdrawal responses applied to the ipsi- and contralateral sides of the face then progressively recovered at post-injection day 9. Symbols represent mean  $\pm$  SEM. Two-way ANOVA followed by Dunnett's post-test for comparisons with baseline (day 0) and Tukey's post-test for comparison between groups: \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$  vs.

day 0; <sup>++</sup>  $P < 0.01$  and <sup>+++</sup>  $P < 0.001$  vs. corresponding vehicle group; <sup>\$\$</sup>  $P < 0.01$  and <sup>\$\$\$</sup>  $P < 0.001$  vs. ipsi-CFA group.

**Figure 3: Facial CFA injection induces anxiety-like behavior in rats.** The anxiety-like behavior was assessed using the Elevated Plus Maze (EPM; A1-3) and Light-Dark Box tests (LDB; B1-3) in two separate cohorts of rats at post-injection days 2-3 and 10-11 (post-injection days 3 and 10 in the graphs) after saline (filled bars) and CFA injections (empty bars). **A.** Bar histograms of the time spent in the open and closed arms (A1), number of open arms crossings (A2) and total distance traveled for 5 min, a measure of locomotor activity, (A3) in the EPM test. Note that CFA-treated rats spent less time within the open arms (and thus more time in closed arms) and entered less into them than saline-treated ones at post-injection day 10 but not at post-injection day 3.  $N = 6$  in each group. **B.** Bar histograms of the latency of the first transition (B1), time spent in the light chamber (B2) and number of transitions (B3) in the LDB test. Note that CFA-treated rats leaved the lit compartment faster, spent less time within the lit compartment and exhibited a smaller number of transitions than saline-treated ones at post-injection day 10 but not at post-injection day 3.  $N$  of saline- and CFA-treated rats at post-injection days 3: 15 and 12, respectively; and at post-injection day 10: 13 and 16, respectively. Bar histograms represent mean + SEM. Symbols indicate replicate values. Two-way ANOVA followed by Tukey's post-test: \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ . (3A1-open arms:  $F_{Drug(1,20)} = 5.9, P = 0.024$ ;  $F_{Time(1,20)} = 6.8, P = 0.017$ ;  $F_{Drug*Time(1,20)} = 9.0, P = 0.007$ ; 3A1-closed arms:  $F_{Drug(1,20)} = 4.5, P = 0.046$ ,  $F_{Time(1,20)} = 1.1, P = 0.3$ ,  $F_{Drug*Time(1,20)} = 5.0, P = 0.037$ ; 3A2:  $F_{Drug(1,20)} = 2.9, P = 0.1$ ,  $F_{Time(1,20)} = 10.9, P = 0.004$ ,  $F_{Drug*Time(1,20)} = 16.7, P < 0.001$ ; 3A3:  $F_{Drug(1,20)} = 6.3, P = 0.020$ ,  $F_{Time(1,20)} = 2.3, P = 0.1$ ,  $F_{Drug*Time(1,20)} < 0.1, P = 0.9$ ; 3B1:  $F_{Drug(1,52)} = 38.2, P < 0.001$ ,  $F_{Time(1,52)} = 85.3, P < 0.001$ ,  $F_{Drug*Time(1,52)} = 50.5, P < 0.001$ ;

3B2:  $F_{\text{Drug}(1,52)} = 71.4, P < 0.001, F_{\text{Time}(1,52)} = 56.0, P < 0.001, F_{\text{Drug*Time}(1,52)} = 74.8, P < 0.001;$

3B3:  $F_{\text{Drug}(1,52)} = 20.0, P < 0.001, F_{\text{Time}(1,52)} = 31.7, P < 0.001, F_{\text{Drug*Time}(1,52)} = 19.4, P < 0.001).$

**Figure 4: Both ipsi- and contralateral rACCs undergo dynamic, bidirectional changes in Fos expression after facial CFA injection.** **A.** Schematic illustrating the location of the rACC also referred to as cingulate cortex area 1 (CG1) (Bregma +3.2 mm). M1, Primary motor cortex; M2, Secondary motor cortex; PrL, Prelimbic cortex; IL, Infralimbic cortex. **B.** Representative photomicrograph of Fos immunoreactivity at low magnification of the ipsilateral rACC (left hemisphere as indicated in A) at post-injection day 3 after saline injection. Scale bar: 50  $\mu\text{m}$ . **C.** Representative photomicrographs of Fos immunoreactivity at high magnification of the ipsilateral rACC (area corresponding to the box in B) at post-injection days 3 (left) and 10 (right) after saline (upper) or CFA injection (lower). Scale bar: 10  $\mu\text{m}$ . **D.** Bar histograms summarizing Fos protein expression (number of Fos<sup>+</sup> neurons in individual rats) in layer II-III and V-VI in saline- (filled bars) and CFA-treated rats (empty bars) of the ipsi- (upper) and contralateral rACCs (lower) at post-injection days 3 (left) and 10 (right). Bar histograms represent mean + SEM. Symbols indicate replicate values. Numbers of saline- and CFA-treated rats at post-injection day 3: 6 and 6, respectively, and at post-injection day 10: 4-5 and 6, respectively. Two-way ANOVA followed by Tukey's post-test: \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ .

**Figure 5: Rostral ACC layer V pyramidal neurons exhibit a depression of both intrinsic excitability and excitatory synaptic transmission 10 days after facial CFA injection.** **A.** Left. Examples of firing responses to 140, 170 and 200 pA current pulses recorded from rACC layer V pyramidal neurons at post-injection day 10 in CFA- and saline-treated rats. Right. Average firing rate vs. current intensity relationships (F-I curves) at post-injection days 3 and 10 in saline- or CFA-treated rats. **B.** Left. Examples of evoked synaptic responses to increasing

stimulus intensities (20, 50 and 100  $\mu$ A) recorded at post-injection day 10 from rACC layer V pyramidal neurons in a CFA- (black traces) and saline-treated rat (grey traces). Right. Average evoked synaptic responses vs. stimulus intensities (log) (input-output curves) at post-injection days 3 and 10 in saline- and CFA-treated rats. C. Left. Examples of synaptic responses to paired stimulations recorded at post-injection day 10 in a CFA- and saline-treated rat. Note that there is a paired-pulse potentiation in the CFA-treated rat but a paired-pulse depression in the saline-treated animal. Right. Average paired-pulse ratios vs. intervals between paired stimulations at post-injection days 3 and 10 in saline- and CFA-treated rats. Symbols in graphs A-C right represent mean  $\pm$  SEM and are as indicated in graph A right. RM two-way ANOVA followed by Fischer's (A) or Newmann-Keuls' (B, C) *post-hoc* tests. CFA vs. Saline: \*  $P < 0.05$ , \*\*  $P < 0.01$ . D. Bar histograms of the rheobase at post-injection days 3 and 10 in saline- and CFA-treated rats. E. Bar histograms of the AMPA/NMDA ratios at post-injection days 3 and 10 in saline- and CFA-treated rats. Bar histograms in D and E represent mean + SEM and are as indicated in D. Two-way ANOVA followed by Newmann-Keuls *post-hoc* test. CFA vs. Saline: \*\*  $P < 0.01$ . Layer 5 pyramidal neurons recorded from saline- and CFA-treated rats are, in A and D, at post-injection days 3 (n = 27 in N = 15 and n = 22 in N = 12, respectively) and 10 (n = 16 in N = 13 and n = 27 in N = 16, respectively) and, in B and C, at post-injection days 3 (n = 21 in N = 15 and n = 25 in N = 12, respectively) and 10 (n = 11 in N = 10 and n = 16 in N = 14, respectively) (n, number of neurons; N, number of animals). In E, the AMPA/NMDA ratio was assessed in 32 neurons.

**Table 1: Properties of individual action potentials recorded at rheobase in the four groups of rats**

Facial injection	Post-injection day 3		Post-injection day 10	
	NaCl	CFA	NaCl	CFA
<b>n (N)</b>	<b>27 (15)</b>	<b>22 (12)</b>	<b>16 (13)</b>	<b>27 (16)</b>
<b>Threshold (mV)</b>	<b>-34.1 ± 1.3</b>	<b>-35.5 ± 0.9</b>	<b>-36.8 ± 1.3</b>	<b>-34.3 ± 1.1</b>
<b>Amplitude (mV)</b>	<b>70.3 ± 2.9</b>	<b>75.6 ± 2.2</b>	<b>76.0 ± 2.1</b>	<b>73.9 ± 2.4</b>
<b>Duration (ms)</b>	<b>5.7 ± 0.3</b>	<b>5.4 ± 0.2</b>	<b>4.9 ± 0.3</b>	<b>4.6 ± 0.3</b>
<b>Time to peak (ms)</b>	<b>1.9 ± 0.1</b>	<b>1.8 ± 0.1</b>	<b>1.7 ± 0.1</b>	<b>1.7 ± 0.1</b>
<b>Area (mV x ms)</b>	<b>141 ± 4</b>	<b>143 ± 4</b>	<b>148 ± 9</b>	<b>133 ± 4</b>

In each group, n is the number of recorded neurons and N, the number of animals.











