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Vagal nerve stimulation blocks interleukin 6-dependent synaptic hyperexcitability induced by lipopolysaccharide-induced acute stress in the rodent prefrontal cortex

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ABSTRACT

The ratio between synaptic inhibition and excitation (sI/E) is a critical factor in the pathophysiology of neuropsychiatric disease. We recently described a stress-induced interleukin-6 dependent mechanism leading to a decrease in sI/E in the rodent temporal cortex. The aim of the present study was to determine whether a similar mechanism takes place in the prefrontal cortex, and to elaborate strategies to prevent or attenuate it.

We used aseptic inflammation (single acute injections of lipopolysaccharide, LPS, 10 mg/kg) as stress model, and patch-clamp recording on a prefrontal cortical slice preparation from wild-type rat and mice, as well as from transgenic mice in which the inhibitor of IL-6 trans-signaling sgp130Fc was produced in a brain-specific fashion (sgp130Fc mice). The anti-inflammatory reflex was activated either by vagal nerve stimulation or peripheral administration of the nicotinic α_7 receptor agonist PHA543613.

We found that the IL-6-dependent reduction in prefrontal cortex synaptic inhibition was blocked in sgp130Fc mice, or – in wild-type animals – upon application sgp130Fc. Similar results were obtained by activating the *"anti-inflammatory reflex"* – a neural circuit regulating peripheral immune response – by stimulation of the vagal nerve or through peripheral administration of the α_7 nicotinic receptor agonist PHA543613.

Our results indicate that the prefrontal cortex is an important potential target of IL-6 mediated trans-signaling, and suggest a potential new avenue in the treatment of a large class of hyperexcitable neuropsychiatric conditions, including epilepsy, schizophrenic psychoses, anxiety disorders, autism spectrum disorders, and depression.

1. Introduction

A number of neurologic and psychiatric conditions including epilepsy, psychotic schizophrenia, and post-traumatic stress disorder are associated with acute, mostly transient, temporal phases characterized by central hyperexcitability which manifests itself with specific clinical features depending on the peculiar nature of the illness (i.e. seizures in epilepsy, psychotic episodes in schizophrenia, low threshold irritability and propensity to aggressive behavior in post-traumatic stress disorder, etc.) (Bauer et al., 2014; Centonze et al., 2005; Wondolowski and Dickman, 2013). Stress often triggers acute hyperexcitable episodes and increases the level of endogenous compounds including steroid hormones, monoamines, and several peptides, mainly of hypothalamic origin.

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A number of these peptides, like the pro-inflammatory cytokine interleukin 6, are produced by "psychogenic" stress, like social challenge (Audet et al., 2011) or sustained changes in circadian rhythmicity (Monje et al., 2011), as well as by systemic stress (Burton et al., 2013; Mays et al., 2012; Merlot et al., 2004; Powell et al., 2009). The relationship between IL-6 and the onset of neuropsychiatric disorder has been previously discussed (Atzori et al., 2012a). For all these reasons we and others focused our work on the central effects of IL-6, finding that this molecule decreases the response of inhibitory GABAergic synapses in the spinal cord (Kawasaki et al., 2008) and mediates the stress-induced decrease of the ratio between synaptic inhibition and excitation (sI/E) in the temporal cortex (Garcia-Oscos et al., 2012).

In this context we looked here for effective methods to reduce the effects of LPS on cortical inhibition, using intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) as a model of acute stress to determine whether the medial prefrontal cortex (mPFC) – an area of the brain whose dysfunctions are at the core of hyperexcitable conditions in the psychiatric spectrum-displays IL-6-dependent hyperexcitability similar to the spinal cord and the temporal cortex.

1.1. Extracellular mechanism of action of IL-6

IL-6 has been found to activate the intracellular Janus kinase/signal transducer activator of transcription (JAK/STAT) cascade using two distinct extracellular mechanisms, named "classical" and "trans-signaling" pathways. In the "classic" pathway, IL-6 binds a composite membrane receptor containing a core represented by the so-called IL-6 receptor (IL-6R) associated with a dimer of the glycoprotein 130 (gp130). Binding of the complex IL-6/IL-6R/gp130, in turn, leads to activation of JAK kinases and subsequent tyrosine-phosphorylation of the gp130 dimer, which in turn activates the STAT cascade. While this specific process has only been identified in a few cellular types including immune cells and hepatocytes, membrane-bound gp130 appears to be present in all nucleated cells and is the signal-transducer for at least 5 other ligands besides IL-6 (Wang et al., 2009).

A second mechanism through which IL-6 can activate membrane-bound gp130 is by previous binding to extracellular IL-6Rs "shed" by immune cells following the activation of the membrane bound proteolytic enzyme ADAM 17 (Rose-John, 2012). In this pathway, named "trans-signaling", the law of mass action linking IL-6, soluble IL-6Rs and their membrane-bound cognate receptor gp130 determines the efficacy of IL-6 in activating the corresponding intracellular cascade. Soluble versions of gp130 are present systemically, (sgp130) sequestering the complex IL-6/IL-6R, and acting as actual IL-6 antagonist (Wolf et al., 2014). Among the tools of this work we have used genetically modified mice (GFAP-sgp130Fc mice) in which a sequence for the production of a dimerized soluble version of gp130 (sgp130Fc) has been placed under the transcriptional control of the promotor for glial fibrillary acidic protein (GFAP), specifically expressed in brain astrocytes (Rose-John, 2012; Yamamoto and Rose-John, 2012). We have recently shown that this manipulation prevents most of the pathological central effects of IL-6 (Campbell et al., 2014).

1.2. The "anti-inflammatory" reflex

We reasoned that since peripheral inflammation contributes to the etiology of psychiatric conditions (Burton and Johnson, 2011; Burton et al., 2013, 2011), attenuation of inflammation may decrease symptoms associated with such spectrum of illnesses. The *anti-inflammatory reflex*, consists in the inhibition of harmful effects of inflammation by inhibiting immune cells through α_7 nicotinic receptors (Tracey, 2002). This phenomenon has been widely documented (Andersson and Tracey, 2012a,b), and has been shown to reduce the effects of immune activation caused by a number of stimuli including LPS (Borovikova et al., 2000). The anti-inflammatory reflex can be activated in at least two ways: by direct administration of α_7 nicotinic agonists, or by vagal nerve stimulation (VNS). VNS has been successfully tested during the last two decades by tens of thousands of patients worldwide without significant side effects as a treatment for pharmacologically untreatable epilepsy (Marras et al., 2013) and major depression (Wani et al., 2013), and is being currently tested as treatment for other conditions like tinnitus (Engineer et al., 2010).

2. Materials and methods

For this study we used 46 wild type mice (C57BL/6J, Charles River), offspring from 3 founding pairs donated by the vivarium of the Instituto de Neurobiologia of the Autonomous University of Mexico (UNAM) in Juriquilla (Queretaro, generous gift from Dr. Raul Paredes), other 20 mice of the same strain, offspring from mice genetically modified in the laboratory of SRJ (for details see this Section 2.4.1), and 112 rats (Sprague Dawley rats, Charles River, Wilmington, MA). Rats were used to routinely perform vagal nerve stimulation (VNS). The use of mice in addition to rats was necessary because of the need to compare wild-type with transgenic animals. Unfortunately we were not successful in performing VNS on mice due to their small size. Otherwise, the general procedures for similar experiments conducted on rat and on mice did not substantially differ between the two species and are reported below.

Given the use of two different species in order to test in order to challenge the generality of our results, we did not pursue a further development of the experimental design with heterozygous mice for the GFAP-sgp130Fc allele, which might have yielded non-conclusive results along with interpretative problem associated with an intermediate concentration of sgp130Fc.

2.1. Brain slices

Animals in their early adolescence, close to reproductive age (25–50-day-old, mean = 35-day-old) were chosen because of their high sensitivity to stress (Harrison and Baune, 2014). Experimental animals were anesthetized with isoflurane (Baxter, Round Lake, IL), sacrificed according to the National Institutes of Health Guidelines, and their brains sliced with a vibratome (VT1200, Leica, Germany) in a cold solution (0–4 °C) containing (mM): 126 NaCl, 3.5 KCl, 10 Glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1.5 $CaCl_2$ and 1.5 $MgCl_2$, titrated at pH 7.4 and saturated with a mixture of 95% O_2 and 5% CO_2 (artificial cerebrospinal fluid, ACSF). Coronal slices (270 μm thickness) were cut from the prefrontal cortex after removal of the olfactory lobes as in previous work (Atzori et al., 2005; Gonzalez-Burgos and Barrionuevo, 2001). Slices from pretreated animals (see next sections in "Section 2") were subsequently incubated in ACSF at 32 °C before being placed in the recording chamber.

2.2. Electrophysiological recording

2.2.1. Patch-clamp recordings

Slices were rapidly transferred to an immersion chamber, where cells with a prominent apical dendrite, suggestive of pyramidal morphology, were visually selected using an upright microscope (BX51, Olympus, Japan) with a 60X objective and an infrared camera system (DAGE-MTI, Michigan City, IN). The recording area in the prefrontal cortex was selected in the intermediate area of the medial aspect (Cg3) from coronal slices of the frontal lobes (Gonzalez-Burgos and Barrionuevo, 2001). In a few trials, synaptic currents and the corresponding parameters were monitored and recorded at 32 °C. While these recordings produced traces with fas-

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ter kinetics, we could not detect other qualitative differences with electrophysiological parameters monitored at room temperature (22 °C), which was chosen to perform the study because it allowed more stable recordings. A −2 mV 100-ms-long voltage pulse was applied at the beginning of every episode to monitor the quality of the recording. Access resistance (10–20 MΩ) was monitored throughout the experiment. Recordings displaying more than 20% change in input or access resistance were discarded from the analysis. All signals were filtered at 2 kHz and sampled at 10 kHz. Further details for the procedures concerning the preparation and electrophysiological recordings, including measurements of the synaptic inhibitory–excitatory ratio and input–output l curves for inhibitory (GABAergic) currents are described in previous work (Atzori et al., 2005; Banerjee et al., 2013; Garcia-Oscos et al., 2012; Gonzalez-Burgos and Barrionuevo, 2001).

2.2.2. Measurement of inhibitory postsynaptic currents

In experiments in which only inhibitory postsynaptic currents (IPSCs) were studied, a holding membrane potential of V_h = −60 mV, was used, with 3–5 MΩ electrodes filled with a solution containing (mM): 100 CsCl, 5 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid K (BAPTA-K), 1 lidocaine N-ethyl bromide (QX314), 1 MgCl2, 10 N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), 4 glutathione, 1.5 ATPMg, 0.3 GTPNa2, 20 phosphocreatine, titrated to pH 7.3, with osmolarity = 270 ± 7 mOsm.

2.2.3. Electrical stimulation of synaptic currents

Electrically evoked postsynaptic currents were measured by delivering one electric stimulus (90–180 μs, 10–50 μA) every 30 s, with an isolation unit A365 (WPI, Sarasota FL), through a glass stimulation monopolar electrode filled with artificial cerebrospinal fluid at approximately 100–200 μm from the recorded neuron. Synaptic responses were monitored at different stimulation intensities before baseline recording. Detection threshold was set at approximately 150% of the SD of the noise (typical noise approximately 4–5 pA, threshold approximately 7–8 pA). The holding voltage was corrected for the junction potential (V_{offset} < 10 mV).

In some experiments, inhibitory and excitatory currents were measured within the same cell. A low-Cl^- intracellular solution was used in which CsCl was lowered to 10 mM, and the remainder 90 mM was substituted with K gluconate, resulting in a theoretical reversal potential for Cl^- close to the hyperpolarized holding potential, in order to minimize the contribution of GABAergic currents (Garcia-Oscos et al., 2012).

IPSCs input–output curves were determined as a function of increasing stimulation intensity. Each point in the input/output (I/O) curves corresponds to averaged responses over 4–10 extracellular electrical responses delivered at the same intensity. For each recording three parameters were extracted: response threshold; initial slope; saturation current. The threshold was the smallest intensity eliciting a non-zero synaptic response. The initial slope was calculated between the first two non-null responses of each curve. The saturation current was the maximum IPSC amplitude observed and typically did not significantly change in the rightmost part of the I/O curve.

2.3. Drugs

Recombinant rat or mouse IL-6 (both from R&D Systems, Minneapolis, Minnesota) was dissolved in phosphate-buffered saline at a concentration of 0.5 μg/mL and was used at 10 ng/mL, in the range of central (up to 2 ng/mL) and systemic (up to 15 ng/mL) concentrations measured after immune challenge (Burton et al., 2011; Girotti et al., 2011). The blocker of the IL-6 trans-signaling pathway (Jostock et al., 2001) soluble glycoprotein 130Fc (sgp130Fc), was produced in the laboratory of S.R.-J. at the Department of Biochemistry at the Christian Albrecht Universität (Kiel, Germany). Lipopolysaccharide (serotype 0127:B8) was purchased from Sigma (St. Louis, Missouri). All other drugs were purchased from Sigma or Tocris (Ellisville, Missouri).

6,7-Dinitroquinoxaline-2,3-dione (DNQX, 10 μM), kynurenate (2 mM) were used in a series of experiments for blocking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor- and *N*-methyl-D-aspartate receptor-mediated currents. Gabazine (20 μM) was used to block $GABA_A$R-mediated currents. After recording an initial baseline for 10–15 min, drugs were bath-applied for 10 min or longer, until reaching a stable condition as defined in *Statistical Analysis* (Garcia-Oscos et al., 2012).

2.4. Animal studies

2.4.1. GFAP-sgp130Fc animals

A vector containing the human glial fibrillary acidic protein GFAP promoter cloned upstream of the optimized soluble glycoprotein 130Fc (sgp130Fc) (Campbell et al., 2014; Rabe et al., 2008) was used for the construction of the transgenic mice expressing sgp130Fc in the central nervous system by astrocytes (GFAP-sgp130Fc mice); a Bcl II/Not I fragment of 5854 bp was isolated from the plasmid and injected into oocytes, which were implanted into foster mothers. The following primers were used for genotyping sgp130Fc mice:

sgp130-Fc-screen forward: 5′-GAG TTC AGA TCC TGC GAC-3′
sgp130-Fc-screen reverse: 5′-TCA CTT GCC AGG AGA CAG-3′

2.4.2. LPS and sgp130Fc injections

Injections of LPS and of sgp130Fc were performed as described previously (Garcia-Oscos et al., 2012). On test day, rats were injected (CMA/100 microinjector, 10 μL Hamilton syringe) intracerebro-ventricularly (i.c.v.) with sterile saline containing 0.1% bovine serum albumin (BSA) (vehicle) or 100 ng sgp130Fc dissolved in 2 μL vehicle. One hour later, rats received IP injection, with sterile saline (0.3 mL) or a dose of LPS (10 mg/kg body weight) which produces an elevation of IL-6 in the prefrontal cortex similar to the concentration of IL-6 applied in the *in vitro* (slice) experiments (Berg et al., 2004; Beurel and Jope, 2009) and were decapitated by a guillotine 8 h after treatment in order to obtain brain slices (Atzori et al., 2001). All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.4.3. VNS

The procedure used for VNS was similar to those used in previous work (Engineer et al., 2010; Nichols et al., 2011). Animals were anesthetized with sodium pentobarbital (50 mg/kg; Sigma–Aldrich Corp., St. Louis, MO, USA). For vagal nerve exposure, a rostro–caudal incision was made in the ventral aspect of the neck on the left side. Using glass probes, muscles were separated and the left cervical-vagus nerve was separated from the carotid artery (see Fig. 1A and B). The vagus nerve was gently guided into a cuff constructed from Micro-Renathane® (0.080" O.D._0.040" I.D.) tubing and braided platinum iridium (.006" diameter) wire with Teflon insulation. The platinum iridium wires lined the inside of the cuff, with the insulation removed to provide conductivity, allowing bipolar stimulation only around the nerve. The platinum-iridium wires from the cuff to the head attachment were threaded subcutaneously along the neck to the top of the skull as described previously (Engineer et al., 2010).

Anesthetized and implanted animals were submitted to stimulation of the vagal nerve (0.5 μA, 1 s stimulation at 10 Hz every 20 s with 500 μs bipolar stimuli during periods indicated as indicated in

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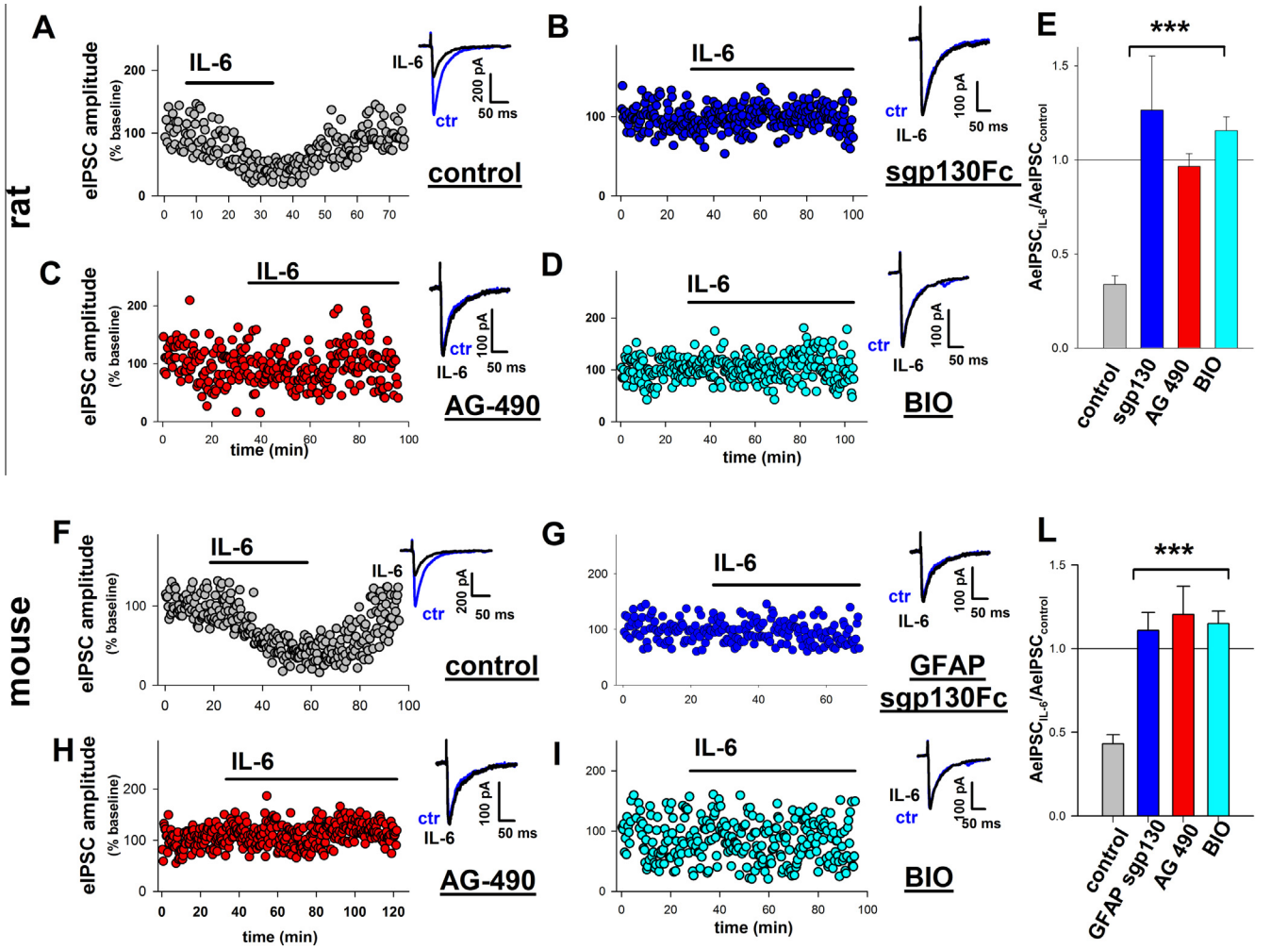

Fig. 1. Effect of IL-6 on the amplitude of IPSCs on rat (above) and mice (below) PFC. (A) Representative example of time course of the IPSC amplitude, before, during, and after the application of IL-6 (10 ng/mL), in control conditions. In the insert on the right is the average of 5 traces immediately before the application of IL-6 (blue trace) or at the maximum effect of IL-6 black trace. In A, B, C, and D, are similar experiment after incubation (20 μg/mL) and superfusion (5 μg/mL) with sgp130Fc, incubation and superfusion with the JAK-STAT inhibitor AG-490 (both 20 μM), or after incubation and superfusion with the glycogen synthase blocker BIO (both 5 μM), respectively. The bar graph in E displays the corresponding changes in IPSC amplitude represented as a ratio (IPSC amplitude in IL-6)/(IPSC amplitude in control) (AIPSCIL-6/AIPSCcontrol), for each condition (control: $n = 10$, other bars: $n = 9$ each experiment). Graphs in F, G, H, I, and J), display the corresponding results from similar experiments in mice, except that G corresponds to slices from GFAP-sgp130Fc mice. Like graph in E, the bar graph in J shows the changes in IPSC amplitude, for each condition ($n = 9$ each experiment). The presence of the IL-6 antagonist sgp130Fc, of the JAK2-STAT3 antagonist AG-490, or of the glycogen synthase blocker BIO prevents the effect of IL-6 in the rat as well as in the mouse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the insert in Fig. 3A, during 10 min, to induce an immediate activation of the anti-inflammatory reflex. Right after VNS animals were treated with an i.p. injection of LPS or saline solution (0.9 g of NaCl/L). After a 10 min break to allow LPS to start its effect, the animal was stimulated again during 45 min before a break of 3 h, at the end of which the animal was sacrificed for brain slicing and subsequent recording. We started VNS prior to the LPS challenge in order to maximize the activation of the anti-inflammatory reflex. While the VNS protocol used had been originally planned for optimizing stimulation of the ascending branch of the vagal nerve, it still substantially activates the descending branch of the vagal nerve.

2.5. Analysis

In our analysis of the postsynaptic current amplitudes, we defined a statistically stable period as a time interval (5–8 min) along which postsynaptic current mean amplitude measured during any two-min assessment did not vary according to Mann–Whitney *U* test (Salgado et al., 2011). All data are expressed as mean ± SEM. The effects of drug application on the PSC amplitude changes were reported as A_{treat}/A_{ctrl}, where A_{treat} and A_{ctrl} are the mean PSC amplitude in treatment and in control respectively. The coefficient of variation was defined as the ratio between standard deviation and mean on samples of 50 evoked responses. Drug effects were assessed by measuring and comparing the different parameters (A_{treat}/A_{ctrl}, inhibitory PSC and excitatory PSC mean amplitudes, or other parameters as indicated) of baseline (control) vs. treatment, with a Mann–Whitney *U*-test. One way ANOVA with Tukey post hoc test was used for comparisons between different groups of cells as in previous work (Salgado et al., 2011). Wilcoxon test was used for comparing between PPRs, and Student *t*-test (paired or unpaired, depending on the experiment) was used for all other comparison. Changes are reported as statistically significant for $p < 0.05$ (* corresponding to $p < 0.05$, ** to $p < 0.02$, *** to $p < 0.01$, not significant: n.s.). All results are shown in the figures. Statistic samples are reported in the figure legends.

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3. Results

3.1. Pharmacology of the IL-6-induced decrease of GABAergic currents in the mPFC

We tested the effects of IL-6 superfusion on visually identified pyramidal cells of layer V in rat medial prefrontal cortex (mPFC). Bath application of IL-6 (10 ng/mL, corresponding to 45 nMol) reversibly decreased inhibitory post-synaptic current (IPSC) amplitude in control (example in Fig. 1A, average in Fig. 1E, gray bar) but not in slices incubated (20 μg/mL) *and* superfused (5 μg/mL) with the sgp130Fc (Fig. 1B, average in Fig. 1E, dark blue bar), consistent with our previous results in the temporal cortex suggesting that IL-6 decreases IPSC amplitude through neuronal trans-signaling (Garcia-Oscos et al., 2012). To determine whether the effect of IL-6 was mediated by activation of the Janus kinase/signal transducer activator of transcription (JAK/STAT) a similar experiment was repeated in slices incubated and superfused with the JAK/STAT blocker tyrphostin (AG-490, 20 μM, example in Fig. 1C, mean in Fig. 1E). Slice incubation and superfusion with AG-490 also prevented the inhibitory effect of IL-6 on IPSCs.

It has been proposed that IL-6-induced inflammation acts through a metabolic cascade operating through the glycogen synthase kinase type 3β (GSK3β) (Li et al., 2013; Zhou et al., 2011), whose allelic variants have been linked to psychiatric disease (Lachman et al., 2007; Lee and Kim, 2011). Incubation and superfusion of brain slices with the GSK3β blocker (2′*Z*,3′*E*)-6-Bromoindirubin-3′-oxime (BIO, 5 μM), also prevented the IL-6-induced IPSC amplitude decrease (example in Fig. 1D, mean in Fig. 1E), suggesting a common mechanisms with IL-6 associated inflammatory mechanisms.

We obtained similar results in recordings from the mPFC of wild type CB57/6 mice, using murine IL-6 (example in Fig. 1F, average in the first bar of Fig. 1L), but not in slices from a strain of CB57/6 mice genetically modified to produce sgp130Fc associated with the promoter of the glial fibrillary acidic protein (GFAP-sgp130Fc mice, example in Fig. 1G, average in the second bar of Fig. 1L). Similar to the finding in rat experiments, the IL-6-induced IPSC amplitude decrease in murine slices was also sensitive to the presence of AG-490 as well as of BIO. Together, all these results suggest that IPSC in the rodent mPFC are vulnerable to the presence of IL-6, that this effect is dependent on an intracellular cascade associated with JAK/STAT and GSK3β, and can be limited or prevented in the presence of a molar excess of sgp130Fc, as expected in the case of an IL-6 action mediated by trans-signaling.

3.2. LPS reduces IPSC in a trans-activation dependent manner

Our previous work has demonstrated that intraperitoneal (IP) administration of the bacterial toxin lipopolysaccharide (LPS) decreases the synaptic ratio between inhibitory and excitatory synaptic currents (sI/E), measured from the same neuron, if measured within 3.5 h after the induction of aseptic inflammation, in rats who received an intracerebroventricular (i.c.v.) injection of saline solution (0.9% NaCl) but not in rats injected with sgp130Fc (Garcia-Oscos et al., 2012). We performed a similar series of experiments in the mPFC of the rat as well as of the mouse. The three traces in Fig. 2A show representative examples of IPSCs and excitatory postsynaptic currents (EPSCs) in rats who have been pretreated with an i.p. injection of saline (left trace), or LPS (10 mg/kg, center and right trace). Each i.p. injection of LPS was preceded by one i.c.v. injection 1 h *before* LPS, with either saline solution (center trace), or the same solution but containing sgp130Fc (1 μg in 1 μL, right trace). The injection of LPS produced a decrease in sI/E if accompanied by the i.c.v. injection of saline but not of sgp130Fc (averages in Fig. 2B). Similar experiments, without the i.c.v. injection were performed on wild-type mice as well as on the GFAP sgp130Fc mice (representative traces in Fig. 2C). The i.p. injection of LPS produced a decrease in IPSC amplitude in wild-type mice but not in GFAP sgp130Fc mice (averages in Fig. 2D).

While these results are consistent with the behavior of the temporal cortex, indicating that the sI/E is sensitive to LPS also in the PFC, we independently assessed GABAergic function in the PFC, by determining stimulus–response (input–output, I/O) curves systematically increasing stimulation intensities and comparing IPSCs saturation levels in same three groups of rats as in the previous experiment (pre-treated with i.p. injection of saline, pre-treated with i.p. injection of LPS and i.c.v., saline, or pre-treated with LPS and i.c.v. sgp130Fc, representative traces in Fig. 2E, I/O curves in Fig. 2F, average saturation current in Fig. 2G). Consistent with previous experiments in the rat, LPS was found to decrease IPSC saturation levels in saline but not in the presence of sgp130Fc. We also determined the effects of i.p. LPS in wild-type and sgp130Fc GFAP mice (representative traces in Fig. 2H. I/O curves in Fig. 2I, average saturation current in Fig. 2L). Consistently with the previous experiment, LPS decreased IPSC saturation current in the wild-type but not in the sgp130Fc GFAP mice. Interestingly, IPSC saturation current was significantly higher in sgp130Fc GFAP mice (with or without LPS), not only compared with LPS-pretreated wild-type, but also with the untreated wild-type (Fig. 2L).

3.3. Activation of the anti-inflammatory reflex blocks LPS-induced hyperexcitability

In order to test the possibility that the activation of the anti-inflammatory reflex attenuated the effects of LPS on central synapses we selected two converging strategies: stimulation of the vagal nerve (VNS), which is known to release acetylcholine on peripheral immune organs like the spleen, or direct systemic activation of nicotinic α_7 receptors. Because of the small size of the mice, surgeries aimed to implant the cuff for VNS on these animals were unsuccessful, whereby in the mouse we only activated the anti-inflammatory reflex by i.p. administration of the nicotinic α_7 agonist PHA543613 (PHA).

3.3.1. Rat

In order to effectively activate the anti-inflammatory reflex concomitantly with the LPS challenge we developed a protocol which mingles the two procedures (Fig. 3A), consisting into implanting a cuff for stimulation of the left vagal nerve and performing VNS (0.5 μA, 1 s stimulation at 10 Hz every 20 s with 500 μs bipolar stimuli) for a total time of 85 min: 10 min before the LPS (i.p., 10 mg/kg) or saline injection, 30 more minutes right after the injection, and a last period of 45 min VNS before a 3 h interval preceding rat sacrifice and brain slicing.

We compared the sI/E ratio obtained from rats i.p. injected with saline solution with those from rats with i.p. LPS injections in 5 conditions: sham (animals with VNS implanted but not stimulated), VNS within 3.5 h after brain slicing, after 3.5 h after brain slicing (no VNS), peripheral (i.p.) administration of PHA543613 (2 mg/kg), or after VNS preceded by bilateral vagotomy of the *ascending* branch of the vagal nerve, in order to assess a possible centripetal effect of VNS. While LPS reduced sI/E ratio in the sham animals, IPSC were not reduced in any of the other conditions (representative traces in Fig. 3B, average in Fig. 3C).

I/O curves and saturation currents were compared between saline-injected rats and rats injected with LPS from the following 4 experimental pre-treatments: sham (LPS), VNS (LPS + VNS), VNS plus bilateral vagotomy (VNS VTX), and PHA543613 (LPS + PHA543613). Only rats pretreated with LPS of the sham group dis-

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played a reduction in saturation current (representative traces in the example in Fig. 3D, I/O curves in Fig. 3E, average saturation currents in Fig. 3F).

3.3.2. Mouse

We compared sI/E ratio and I–O curves from wild-type mice i.p. injected with saline solution, or with LPS without or with the nicotinic α_7 agonist PHA. LPS administration decreases sI/E ratio in control but not in the presence of PHA (representative traces in Fig. 4A, mean sI/O in Fig. 4B). Consistently with these results, I/O curves possessed lower saturation values in LPS-administered animals, but not after PHA pretreatment (representative traces in Fig. 4C, I/O curves in Fig. 4D, and mean saturation curves in Fig. 4E).

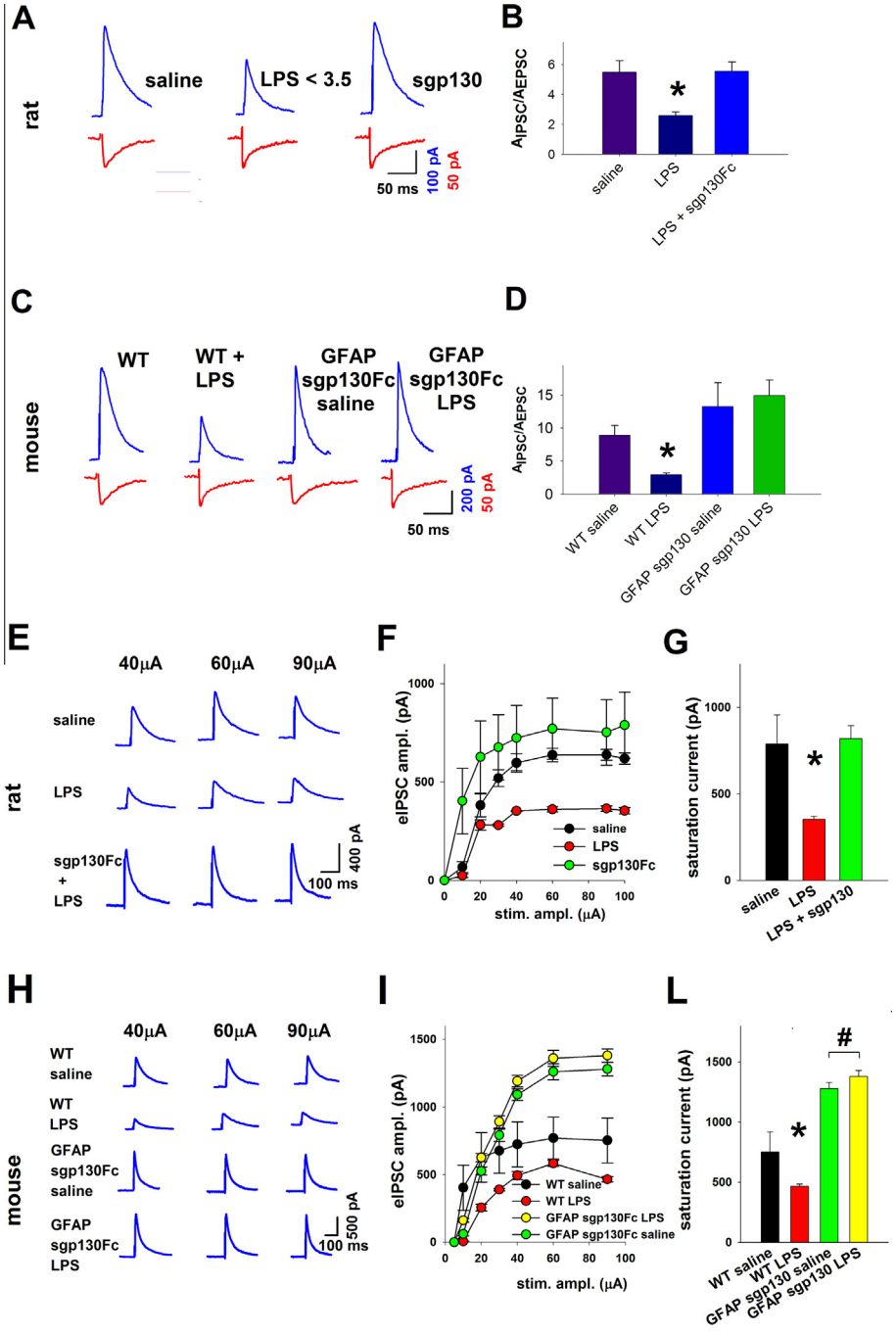

Fig. 2. Effect of LPS. (A) Representative examples of the sI/E ratio in the rat PFC after injection of saline solution i.p. and i.c.v. (left), or of LPS (i.p. 10 mg/kg) and i.c.v. saline (center) or i.c.v. sgp130Fc (right, 1 μg, 1 h before LPS injection). (B) Average effect (i.p. saline and i.c.v. saline, $n = 5$ first bar; i.p. LPS in the absence of sgp130Fc, $n = 9$, second bar, or in its presence, $n = 10$, third bar). (C) Representative traces showing the effects of LPS on the sI/E ratio in the PFC of wild-type mice (1st and 2nd traces) or in GFAP-sgp130Fc mice (3rd and 4th traces). (D) Average sI/E ratio in the corresponding sample ($n = 5$, 9, 11, and 12, each bar respectively). LPS only decreases the ratio between inhibition and excitation in WT but not in GFAP sgp130Fc mice. (E) Representative traces at different stimulation intensities, (F) input–output (I–O) curves ($n = 5$), and (G) average saturation currents, respectively, displaying the effect of LPS in the PFC of the rat in the absence or in the presence of sgp130Fc in the recording solution (same animal treatment as in A and B, $n = 5$ each experiment, same sample as F). LPS is accompanied by a decrease in the general IPSC amplitude and its saturation levels in but not in the presence of sgp130Fc. (H) Representative traces, (I) I–O curves ($n = 5$), and (J) average saturation levels displaying the effect of LPS on WT mice (upper two rows in H) or in GFAP-sgp130Fc mice (lower two traces in H). LPS injection only decreases IPSCs in WT mice but not in GFAP-sgp130Fc mice. IPSCs saturation levels of both saline and LPS injected animals are significantly higher than saline injected animals ($n = 5$ each experiment, same sample as I).

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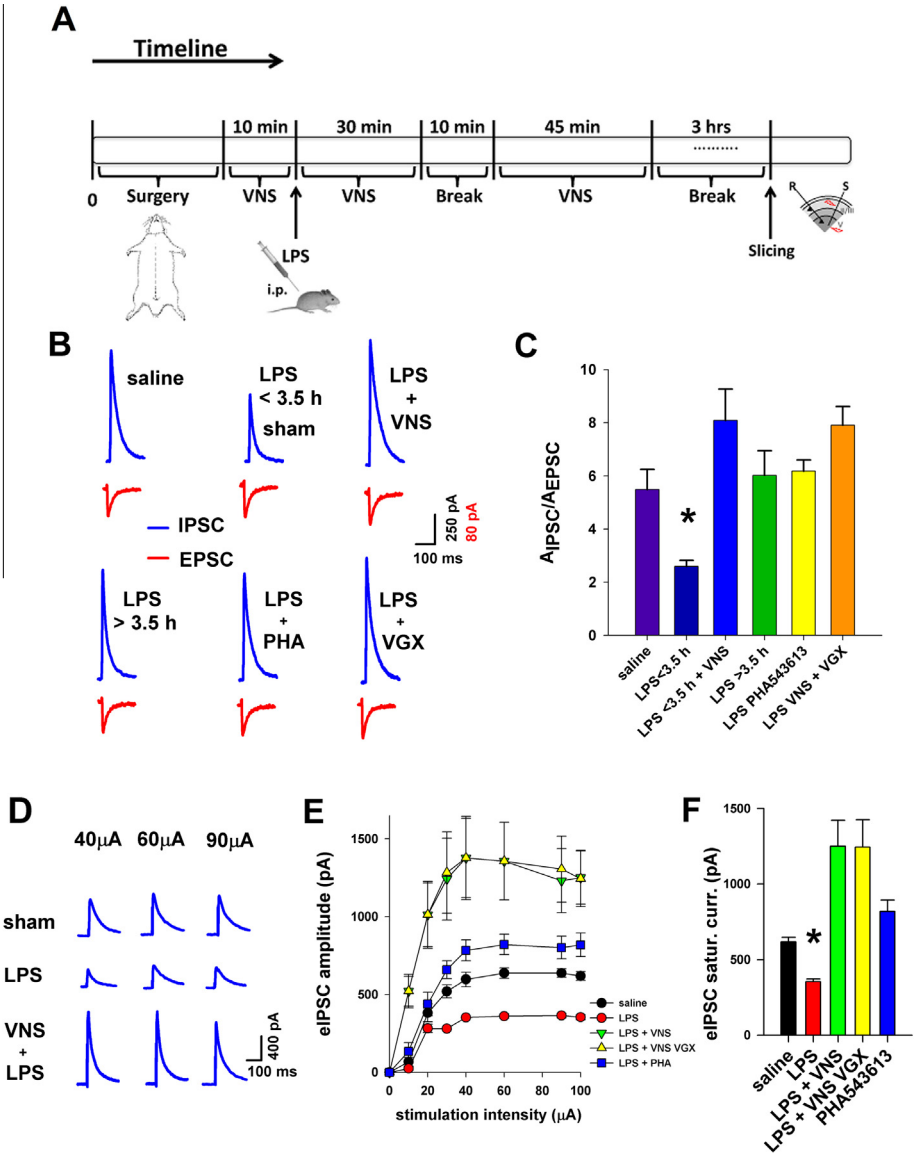

Fig. 3. Effects of VNS. (A) Anesthetized and implanted animals were submitted to stimulation of the vagal nerve (0.5 μA, 1 s stimulation at 10 Hz every 20 s with 500 μs bipolar stimuli during periods indicated as indicated in the insert in Fig. 3, during 10 min. Right after VNS animal were treated with an i.p. injection of LPS or saline solution (0.9 g of NaCl/L). After a 10 min break the animal was stimulated again during 45 min before a break of 3 h, at the end of which the animal was sacrificed for brain slicing and subsequent recording. (B) and (C) Representative traces and average effect of LPS injection on the sI/E ratio under different conditions in the PFC of the rat, respectively. While the sI/E ratio was decreased in recordings from animals during the first 3.5 h period after slicing (2nd bar, $n = 11$; 1st bar is control: $n = 8$), the same treatment after VNS was associated with a non-decreased sI/E ratio (3rd bar, $n = 13$). A >3.5 h wait also was associated with non-decreased sI/E ratio (4th bar, $n = 6$), as well the pre-treatment with the i.p. injection nicotinic α_7 agonist PHA543613 (2 mg/kg, $n = 10$) 1 h before LPS (5th bar). A cut of the ascending branch of the left vagus nerve did not modify the result (6th bar, $n = 12$). (D–F) Representative traces and average effect of LPS injection on I–O curves and current saturation levels under different conditions in the PFC of the rat. While in control conditions LPS injection reduces currents and IPSC saturation level, the same experiment after VNS, with or without vagotomy, or after pre-treatment (i.p. injection 1 h before LPS injection) with the nicotinic agonists PHA543613 (2 mg/kg) does not decrease inhibitory currents and their saturation levels ($n = 5$ for each condition, data for saline and LPS alone are reported from the previous figure for comparison).

4. Discussion

A number of neuropsychiatric conditions including schizophrenia (Behrens et al., 2008), anxiety disorders (Jones and Thomsen, 2013), but also depression (Hashmi et al., 2013), disorders of the autism spectrum (Wei et al., 2013) have been associated with increased level of IL-6 on one hand, and impairment of the GABAergic system on the other (Atzori et al., 2012b). Although the role of IL-6 in epilepsy appears to be complex, as some early reports identified a protective effect of IL-6 in brain inflammation (Penkowa et al., 2001), a consensus has been generated by later studies which highlighted mostly epileptogenic effects of this cytokine (Samland et al., 2003; Vezzani et al., 2008) accompanied by increased excitability (Nelson et al., 2012). In the attempt to find a causal correlation linking this particular cytokine with a dysfunction of the major inhibitory cortical network, we first sought to determine the effect of IL-6 on GABAergic transmission in the PFC, a critical area in the pathophysiology of most psychiatric conditions.

We found, in the rat as well as in the mouse, that IL-6 reversibly decreases GABAergic currents in the mPFC in a JAK/STAT-, GSKβ_3-, and gp130-dependent manner, suggestive of a trans-activation pathway for IL-6. In the same two species we also found that systemic administration of LPS also induces central acute hyperexcitability, measured as decrease in sI/E accompanied by a reduction in the saturation levels of I/O curves, and that this phenomenon is also blocked in the presence of sgp130Fc. All these data are consistent with our previous work in the temporal cortex (Garcia-Oscos

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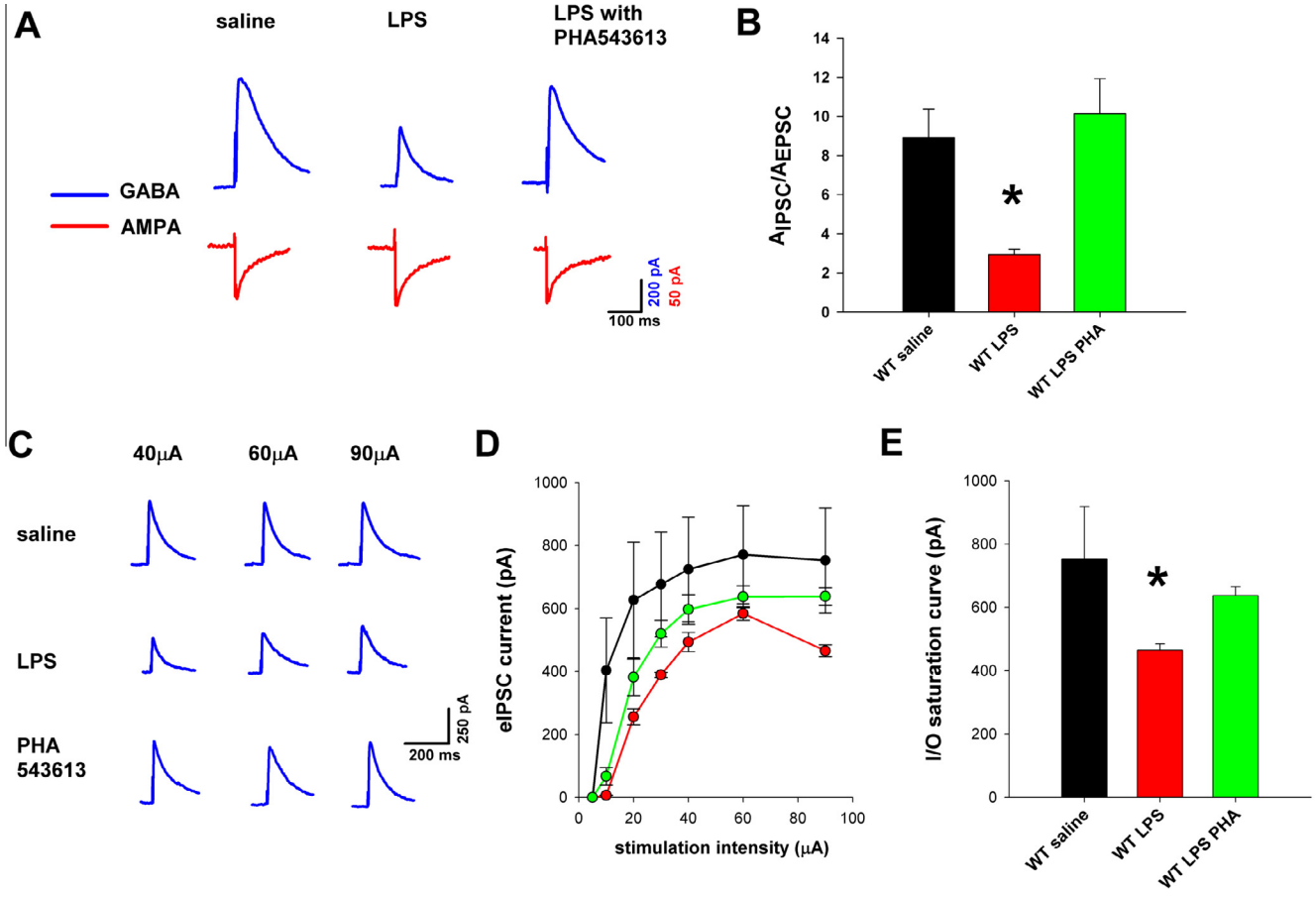

Fig. 4. Effect of PHA543614 in the mouse. (A) Representative recordings and average data displaying sI/E after i.p. injection of different solutions: saline (left), LPS in the absence of the nicotinic α_7 receptor agonist PHA 543613 (center), or in its presence (right). (B) Average sI/E: saline ($n = 6$, 1st bar), LPS alone ($n = 10$, 2nd bar), and LPS +PHA543614 ($n = 10$, 3rd bar). (C) Representative traces at different intensities (D) I–O curves ($n = 5$), and (E) I–O curve saturation levels for IPSCs in the PFC of the mouse. The presence of PHA543613 prevents the decrease of inhibition or I–O currents and saturation levels induced by LPS ($n = 5$ for each condition, same sample as D, data for saline and LPS alone are shown again for comparison).

et al., 2012), suggesting that the stressed prefrontal cortex is similarly subject to an increase in excitability, as well as on other brain areas (Campbell et al., 2014), where we showed that trans-signaling is the leading IL-6-mediated pathogenic mechanism. While our data suggest that the "classic" IL-6 pathway mediated by membrane receptors composed by the complex IL-R/gp130 (present in astrocytes, microglia, and other immune cells resident in the brain and/or in the blood brain barrier), plays at most a minor role in the LPS-mediated increase in synaptic excitability, caution should be used in generalizing the validity of our data. In fact, given the pleiotropy of its effects, we cannot exclude that increases in the brain concentration of IL-6 may have different – even opposite – effects on brain excitability as a result of longer (chronic of subchronic) time frames or for different types of stressors. The results from this and former studies do not exclude the possibility that cortical GABAergic transmission may be critically subject to additional or alternative pathophysiological modulation by other stress-related molecules, including corticotropin releasing factor CRF) and/or glucocorticoids, in a time window different from that associated with acute LPS injection (Andersson and Tracey, 2012b). The theoretical existence of short-loop (intra-hypothalamic or otherwise centrally-mediated) feedback among group of cells either releasing, and/or modulated by, IL-6, CRF, and glucocorticoids further increases the intricacy of the CNS stress molecular network, and, by consequence, the interpretation of ours and any stress-related behavioral experiments. Nonetheless, we have previously shown that sgp130Fc does not appreciably inhibit LIF, OSM, CNTF and IL-27 (Jostock et al., 2001; Scheller et al., 2005). The complex of IL-11 /sIL-11R would be inhibited by sgp130Fc but so far, no soluble IL-11R has been detected. Therefore we believe that the IL-6/sIL-6R complex is responsible for the LPS effects observed. While ours and others data point at IL-6 as a critical factor in the etiology of neuropsychiatric disease (Atzori et al., 2012b), a possibility remains that other gp130 agonists like leukemia inhibiting factor or IL-11 may additionally or alternatively contribute to stress-induced conditions, particularly in the relatively non-specific LPS model of acute stress. Whether or not this is the case, a corollary of our results is that conditions resulting from stress-induced hyperexcitability might be treated with i.c.v. administration of a molar excess of sgp130Fc, or other compounds chelating soluble molecules or complexes which – like the IL-6/IL-6R complex – activate membrane bound gp130 in the brain. Although this specific application had never been tested before, a similar strategy has been successfully used in recent studies, peripherally (Braun et al., 2013; Chalaris et al., 2012; Zhang et al., 2013), centrally (Burton and Johnson, 2011; Burton et al., 2013, 2011; Campbell et al., 2014), as well as in the reduction of neuroinflammation and sickness behavior following LPS peripheral injections and in the inhibition of HPA response to various types of stress (Girotti et al., 2013).

In the search of treatments that do not require direct access to the CNS, we investigated the possibility that activation of the *anti-inflammatory reflex* (Borovikova et al., 2000; Tracey, 2002) may decrease central consequences of peripheral inflammation. We found that both in rat and mouse, administration of the nicotinic α_7 agonist PHA successfully reduces the LPS-induced mPFC hyperexcitability, while VNS prevents it to an even larger extent, at least

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in the rat. Similar results were obtained after severing both ascending branches of the vagal nerves, indicating that this effect of VNS was carried out mainly or exclusively by the descending branch of the vagal nerve.

A causal relationship between IL-6 and schizophrenia had been suggested in earlier studies suggesting that IL-6 mediates specific oxidative damage to a set of PFC GABAergic neurons containing the Ca^{2+}-binding protein parvalbumin (PV) (Behrens et al., 2007, 2008; Behrens and Sejnowski, 2009; Dugan et al., 2009). Recent work in animal models shows that the development of PV+ neurons, as well as that of other classes of local inhibitory interneurons, is – in fact – impaired by pharmacological and other types of stressors, particularly during specific stages of adolescence (Caballero et al., 2013a,b; Cass et al., 2013; Feleder et al., 2010; Tseng et al., 2008). Our data provide an additional or alternative interpretation causally linking acute stress to the trigger of a psychotic episode. IL-6 has also been specifically related with the onset of depression (Sukoff Rizzo et al., 2012), which in turn has been linked to a GABAergic impairment since the 80's (Bajbouj et al., 2006; Drugan et al., 1989). Our data offer a possible direct explanation for the wide use and effectiveness of $GABA_A$ receptor ($GABA_AR$) enhancers in the treatment of the symptoms of clinical depression, which is problematic because of their non-specific sedative and desensitizing effects (Chen et al., 2008). Similarly, a sustained increase in the level of IL-6 induced by stress, and a concomitant decrease in $GABA_AR$ function in prefrontal areas may explain – at least in part – the results obtained from sufferers of post-traumatic stress disorder (Geuze et al., 2008; Rossi et al., 2009). Results from IL-6 knock-out animals (De Sarro et al., 2004) showing a decrease in the threshold for seizure, suggest that the levels of IL-6 control not only the expression and effectiveness of postsynaptic $GABA_ARs$, but also, indirectly, the equilibrium between excitatory and inhibitory synapses, which is continuously adjusted to maintain a dynamic equilibrium at the base of the adaptive synaptic plasticity (Turrigiano, 2008).

5. Conclusions

Altogether, our results suggest that (1) sgp130Fc may be effective in reducing the detrimental effects associated with a stress-induced increase in IL-6, and that (2) a cholinergic-induced reduction in peripheral immune activation could also be a viable, effective, and minimally invasive therapy for at least some types of hyperexcitable condition. These considerations open an additional conceptual framework for the interpretation on the so far incompletely described synaptic mechanisms behind the therapeutic effect of VNS in the treatment of epilepsy and depression, and a novel avenue in the treatment of neuropsychiatric conditions including schizophrenic psychoses, depression, post-traumatic stress disorder and other anxiety-related syndromes.

While our data support a critical role between IL-6 and the GABAergic system in the etiology of a large array of neuropsychiatric disorders (Atzori et al., 2012b; Hines et al., 2012), many questions remain open about the nature of the interplay involving stress, IL-6, and GABAergic impairment. Further work will be needed to determine the precise relationship between type of stress and the nature and anatomic and synaptic location of the GABAergic impairment, and whether the critical central effects of IL-6 are associated with peripheral or centrally produced IL-6 (Girotti et al., 2013; Sallmann et al., 2000).

Author contributions

FGO and MH performed all patch-clamp slice experiments, FGO, DP, and MSB performed VNS implant surgeries, MH and LCD performed bilateral cervical vagotomies, DC supervised breeding and supplied animals, FGO supervised and performed i.c.v. injections and cannula implants, DL, MH, and FGO performed data analysis, SDM, JN, FGO and DC genotyped genetically modified mice, SRJ developed and supplied the GFAP-sgp130Fc mice. FGO, SDM, RS, HS, SRJ, and MPK, gave intellectual contributions. FGO and MA developed the ideas of the project and the experimental plan, interpreted the results, and wrote the manuscript.

Funding and disclosure

As disclosure of possible conflict of interest, we would like to report that Dr. Rose-John is an inventor on the patent describing the function of sgp130Fc. He is also a shareholder of the CONARIS Research Institute (Kiel, Germany), which is commercially developing sgp130Fc as a therapy for inflammatory diseases. All other authors have no financial interests or potential conflicts of interest and no relevant funding.

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