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Activation of the Anti-Inflammatory Reflex Blocks Lipopolysaccharide-Induced Decrease in Synaptic Inhibition in the Temporal Cortex of the Rat

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Stress is a potential trigger for a number of neuropsychiatric conditions, including anxiety syndromes and schizophrenic psychoses. The temporal neocortex is a stress-sensitive area involved in the development of such conditions. We have recently shown that aseptic inflammation and mild electric shock shift the balance between synaptic excitation and synaptic inhibition in favor of the former in this brain area (Garcia-Oscos et al., 2012), as well as in the prefrontal cortex (Garcia-Oscos et al., 2014). Given the potential clinical importance of this phenomenon in the etiology of hyperexcitable neuropsychiatric illness, this study investigates whether inactivation of the peripheral immune system by the "anti-inflammatory reflex" would reduce the central response to aseptic inflammation. For a model of aseptic inflammation, this study used i.p. injections of the bacterial toxin lipopolysaccharide (LPS; 5 μM) and activated the anti-inflammatory reflex either pharmacologically by i.p. injections of the nicotinic α_7 receptor agonist PHA543613 or physiologically through electrical stimulation of the left vagal nerve (VNS). Patch-clamp recording was used to monitor synaptic function. Recordings from LPS-injected Sprague Dawley rats show that activation of the anti-inflammatory reflex either pharmacologically or by VNS blocks or greatly reduces the LPS-induced decrease of the synaptic inhibitory-to-excitatory ratio and the saturation level of inhibitory current input–output curves. Given the ample variety of pharmacologically available α_7 nicotinic receptor agonists as well as the relative safety of clinical VNS already approved by the FDA for the treatment of epilepsy and depression, our findings suggest a new therapeutic avenue in the treatment of stress-induced hyperexcitable conditions mediated by a decrease in synaptic inhibition in the temporal cortex.

Key words: vagal nerve stimulation; VNS; GABA; PHA543613; nicotinic α7

Stress, together with genetic predisposition, is a major component in the etiology of neuropsychiatric disease (Hains and Arnsten, 2008; Craig, 2010). Many decades of basic and clinical research suggest that illness, physical injury, environmental challenge, and psychological stress share a converging pathway leading, in vulnerable individuals, to mental disease through common pathological mechanisms involving activation/hyperactivation of the neuroendocrine axis (Herman, 2010) and of the autonomic system (Goldstein, 2010) as well as inflammation (Leonard and Song, 2010).

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The temporal cortex (TC) is a stress-sensitive brain area that has been implicated in the etiology of numerous neuropsychiatric illnesses, including depression (Van Tol et al., 2010; Kroes et al., 2011; Lai and Wu, 2012; Frick et al., 2013), anxiety disorders (Cassimjee et al., 2010; Inkster et al., 2011), and schizophrenic psychoses (Jalbrzikowski et al., 2013; Fulham et al., 2014; Pietersen et al., 2014a,b; Sellmann et al., 2014). The exquisite sensitivity of human TC to psychosocial stress (Allendorfer and Szaflarski, 2014; Allendorfer et al., 2014) is underscored by the area-specific finding of increased endoplasmic reticulum stress proteins in brains from suicide subjects (Bown et al., 2000). Temporal cortical areas are also specifically vulnerable to inflammation following typhoid vaccination, as shown by positron emission tomography (Harrison et al., 2014).

One of the hallmarks of schizophrenia is the impairment of a large class of interneurons releasing γ-aminobutyric acid (GABA; Deidda et al., 2014; Pietersen et al., 2014b; Schmidt and Mirnics, 2014), which is responsable, at least in the juvenile and adult mammal, for synaptic inhibition. The relationship between stress and synaptic impairment has not been fully unraveled. It is known that different types of acute stress activate a systemic inflammatory cascade (Steptoe et al., 2007; Munhoz et al., 2008), which in turn induces central effects, such as a decrease in the ratio between synaptic inhibition and synaptic excitation (sI/E; Beattie et al., 2002; Stellwagen and Malenka, 2006; Garcia-Oscos et al., 2012), of which a reduced GABAergic signaling is an important component. Because this phenomenon has a large pathogenic potential in the etiology of hyperexcitable neuropsychiatric conditions such as schizophrenic psychoses and epilepsy (Atzori et al., 2012), this study investigates whether the activation of a peripheral cholinergic process, referred to as the "anti-inflammatory reflex" (Tracey, 2002; Wang et al., 2003; Oke and Tracey, 2009), has the ability to contain the stress-induced central reduction in GABAergic signaling.

The current study uses aseptic inflammation as a rat model of acute stress, consisting of a single, systemic injection of the bacterial toxin lipopolysaccharide (LPS), which has previously been shown to be effective in reducing GABAergic currents (Atzori et al., 2012; Garcia-Oscos et al., 2012). Excitatory synaptic currents and their ratios were monitored with patch-clamp recording in brain slices from previously treated animals. The anti-inflammatory reflex was activated either indirectly by stimulation of the left vagal nerve (VNS), which releases endogenous acetylcholine through activation of its descending (motor) branch, or directly through activation of peripheral nicotinic α_7 receptors, which mediate the anti-inflammatory reflex. The results show that either VNS or systemic injection of a nicotinic α_7 agonist suffices to block or greatly reduce the decrease of synaptic inhibition and the corresponding shift in the s/IE produced by systemic LPS.

MATERIALS AND METHODS

Brain Slices

Sprague Dawley rats, 25–50 days old (mean ≈35 days old), were housed two to four per cage in the facilities of the vivarium at 22°C and about 50% humidity subject to a 12-hr light/dark cycle and handled by trained laboratory personnel to minimize stress (cage cleaning, displacement between cages, etc.). Animals were anesthetized with isoflurane (Baxter, Round Lake, IL) and sacrificed according to NIH guidelines, and their brains were sliced with a vibratome (VT1200; Leica, Wetzlar, Germany) in a cold solution (0–4°C) containing (in 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 most caudal one-fourth of the brain after removal of the occipital convexity as previously described (Atzori et al., 2001, 2005). Slices from pretreated animals (as described in the following sections) were subsequently incubated in ACSF at 32°C before being placed in the recording chamber.

Patch-Clamp Recordings

Slices were rapidly transferred to an immersion chamber where layer 2/3 cells were visually selected by using an upright microscope (BX51; Olympus, Tokyo, Japan) with a ×60 objective and an infrared camera system (DAGE-MTI, Michigan City, IN). Although the selection of large, triangular cell bodies with a prominent apical dendrite was suggestive of pyramidal morphology, the sporadic picking of local GABAergic interneurons (which represent <10% of the local neurons) cannot be ruled out. A 2-mV 100-msec-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. All experiments were performed at room temperature (22°C). Recordings that displayed 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 of the procedures with regard to the preparation and electrophysiological recordings, including measurements of the synaptic inhibitory–excitatory ratio and input–output (I/O) curves for inhibitory (GABAergic) currents, have been described elsewhere (Kawaguchi, 1995; Atzori et al., 2001, 2005; Garcia-Oscos et al., 2012).

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 (in mM) 100 CsCl, 5 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid K, 1 lidocaine N-ethyl bromide, 1 $MgCl_2$, 10 N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), 4 glutathione, 1.5 ATPMg, 0.3 $GTPNa_2$, and 20 phosphocreatine titrated to pH 7.3, with osmolarity of 270 ± 5 mOsm.

Electrical Stimulation of Synaptic Currents

Electrically evoked postsynaptic currents were measured by delivering one electrical stimulus (90–180 μsec, 10–50 μA) every 30 sec with an isolation unit A365 (WPI, Sarasota, FL) through a glass stimulation monopolar electrode filled with ACSF at approximately 100–200 μm from the recorded neuron. Synaptic responses were monitored at different stimulation

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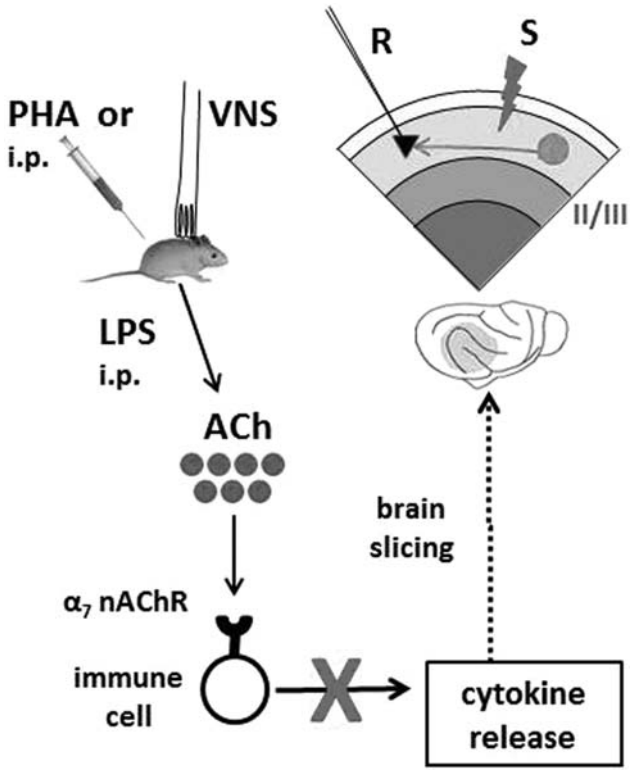

Fig. 1. Schematic of the time sequence of VNS. VNS (one 500-msec train at 5 Hz every minute) was begun 10 min before the i.p. injection of LPS (5 μM) and continued during an interval of 75 min, with a 10-min break halfway through. After a 3-hr interval (at the end of which the effect of LPS is maximal), the animal was sacrificed, and synaptic currents were recorded during the next several hours. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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, to minimize the contribution of GABAergic currents (Garcia-Oscos et al., 2012).

IPSC I/O curves were determined as a function of increasing stimulation intensity. Each point in the I/O curves corresponds to averaged responses over four to 10 extracellular electrical responses delivered at the same intensity. For each recording, three parameters were extracted, response threshold, initial slope, and saturation current. The threshold was the smallest intensity eliciting a nonzero synaptic response. The initial slope was calculated between the first two nonnull 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.

Drugs

LPS (serotype 0127:B8) was purchased from Sigma (St. Louis, MO). All other drugs were purchased from Sigma or Tocris (Ellisville, MO). 6,7-Dinitroquinoxaline-2,3-dione (10 μM) and kynurenate (2 mM) were used 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 $GABA_A$ receptor ($GABA_AR$)-mediated currents. After recording of an initial baseline for 10–15 min, drugs were bath applied for 10 min or longer until they had reached a stable condition as defined in Statistical Analysis (Garcia-Oscos et al., 2012).

Animal Procedures

Rats were anesthetized with isoflurane, and body temperature and respiration were maintained at physiological levels. All procedures were performed in accordance with the NIH *Guidelines for the care and use of laboratory animals*. Rats received an i.p. injection of sterile saline (0.3 ml) or a dose of LPS (10 mg/kg of body weight) and were decapitated with a guillotine 8 hr after treatment to obtain brain slices (Atzori et al., 2001).

The procedure used for VNS was similar to procedures described elsewhere (Engineer et al., 2010; Nichols et al., 2011). Animals were anesthetized with sodium pentobarbital (50 mg/kg; Sigma). For vagal nerve exposure, a rostrocaudal incision was made in the ventral aspect of the neck on the left side. Muscles were separated with glass probes, and the left cervical vagus nerve was separated from the carotid artery. The vagus nerve was gently guided into a cuff constructed from Micro-Renathane (0.080 in. o.d., 0.040 in. i.d.) tubing and braided platinum iridium (0.006 in. 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 subjected to stimulation of the vagal nerve (0.5 μA, 1-sec stimulation at 10 Hz every 20 sec with 500-μsec bipolar stimuli for 10 min) to induce an immediate activation of the anti-inflammatory reflex. Immediately after VNS, animals were treated with an i.p. injection of LPS or saline solution (0.9 g NaCl/liter). After a 10-min break to allow the LPS to start its effect, animals were again stimulated for 45 min before a break of 3 hr, at the end of which animals were sacrificed for brain slicing and subsequent recording. VNS was started prior to the LPS challenge to maximize activation of the anti-inflammatory reflex. Although the VNS protocol that was 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. A sketch of the experimental sequence of the protocol is shown in Figure 1. F1

Statistical Analysis

For analysis of the postsynaptic current amplitudes, a statistically stable period was defined as a time interval

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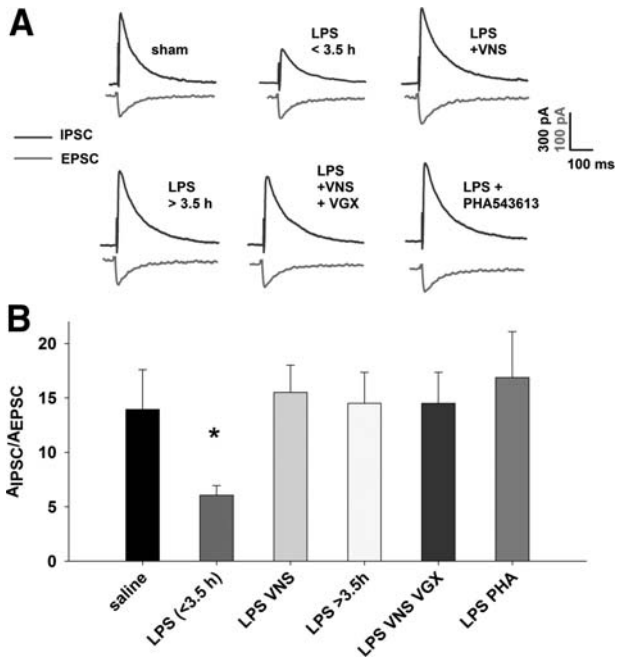

Fig. 2. Activation of the "anti-inflammatory reflex" blocks the LPS-induced reduction in synaptic ratio between inhibition and excitation. **A:** Single-cell patch-clamp recordings display representative traces of inhibitory currents (blue) and excitatory currents (red) in the following experimental settings: implanted but nonstimulated vagal nerve stimulator in i.p. saline-injected animals (sham, upper left traces); nonstimulated vagal nerve stimulator in LPS-injected animal recorded before 3.5 hr after LPS injection (LPS < 3.5, upper center traces). After LPS injection and VNS (LPS + VNS, protocol given in Fig.1, upper right traces), LPS-injected recorded more than 3.5 hr after LPS injection (LPS > 3.5 hr, lower left traces), LPS injection and VNS with vagotomy of the ascending branch of the stimulated (left) vagus nerve (LPS + VNS + VGX, lower center traces), and LPS injection following i.p. injection of the nicotinic α_7 receptor agonist PHA543613 (LPS + PHA543613, lower-right traces). **B:** Average ratio between inhibitory and excitatory synaptic currents for the conditions listed above. $^{\star}P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(5–8 min) during which postsynaptic current mean amplitude measured during any 2-min assessment did not vary according to the Mann-Whitney U test (Salgado, et al., 2011a,b). All data are expressed as mean ± SEM. The effects of drug application on the PSC amplitude changes are reported as A_{treat}/A_{ctrl}, where A_{treat} and A_{ctrl} are mean PSC amplitude in treatment and in control, respectively. 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's post hoc test was used for comparisons between different groups of cells (Salgado et al., 2011a). Student's *t*-test (paired or unpaired, depending on the experiment) was used for all other comparisons. Changes are reported as statistically significant at $P < 0.05$. All results are shown in the figures, and statistical samples are reported in the figure legends.

RESULTS

To determine how the anti-inflammatory reflex modified the synaptic response to LPS in the TC, we measured electrically evoked excitatory and inhibitory postsynaptic currents (eEPSCs and eIPSCs) with two different patch-clamp recording assays on slices from pretreated animals. The first assay determined the ratio between synaptic inhibition and synaptic excitation within a single neuron with a recording intracellular solution with markedly different reversal potential for $GABA_AR$-mediated currents vs. glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated currents, whereas N-methyl-D aspartate receptor-mediated currents were blocked pharmacologically. As described previously, recording at the reversal potential for one receptor minimizes its contribution while allowing measurement of the synaptic response for the other. The ratio between eIPSCs and eEPSCs was calculated off-line (see Materials and Methods). The second assay determines stimulus–response (I/O) curves for eIPSC and for increasing stimulation currents, as described elsewhere (see Materials and Methods).

Activation of the Anti-Inflammatory Reflex Blocks the LPS-Induced Reduction in the sI/E Ratio

To activate the anti-inflammatory reflex, a platinum stimulator (cuff) was implanted around the left vagal nerve, and the following protocols were used: 1) stimulation of the disconnected electrode in i.p.-injected animals (sham); 2) stimulation of the disconnected electrode in i.p. LPS-injected animals; and 3) stimulation of the fully connected electrode in i.p. LPS-injected animals (LPS + VNS). The averages of 10 or more representative eIPSCs (blue) and eEPSCs (red) are shown in the upper traces in Figure 2A. eIPSCs and eEPSCs are represented F2
by outward and inward currents recorded, respectively, at a resting potential, the Nernst potential for AMPAR- and $GABA_AR$-mediated currents, respectively. As expected from previous work, LPS injection yielded a significant and specific decrease of eIPSC amplitude, whereas eEPSC amplitude was, on average, unchanged in recordings performed within the first 3.5 hr after sacrificing the experimental animal. The effect of LPS is shown in the difference between the first and the second couple of traces (Fig. 2A, left and center), displaying a typical proportion for such currents (Fig. 2B, average in the black and red bars; n = 6 and n = 9, respectively; $P < 0.02$, unpaired Student's *t*-test). VNS resulted in a complete block of the depressing effect of LPS, as shown in the upper right couple of traces in Figure 2A (nonsignificantly different [n.s.] compared with sham; n = 12; Fig. 2B, average in the green bar) under the same recording conditions. Recordings were started more than 3.5 hr after sacrificing the animal (LPS > 3.5 hr; n = 9) and displayed an sI/E ratio similar to control (sham) values for sI/E, as shown in the representative traces at lower left in Figure 2A (Fig. 2B, average in the yellow bar).

To answer the question of whether the ascending branch of the vagal nerve was responsible for at least part

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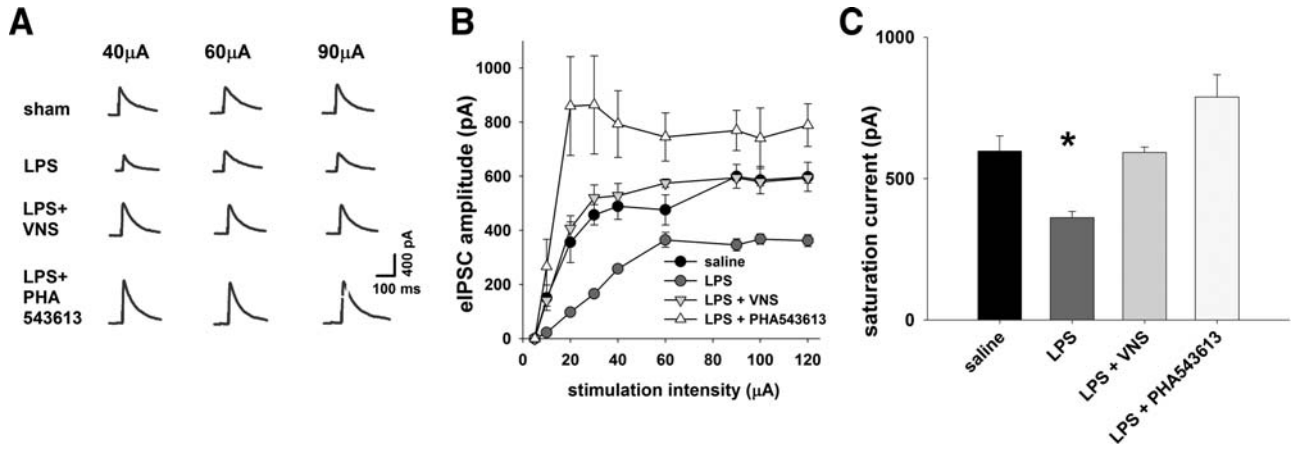

Fig. 3. Activation of the anti-inflammatory reflex blocks the LPS-induced reduction of the saturation levels of I/O curves of inhibitory currents. **A:** Representative traces of eIPSCs obtained from slices at the indicated stimulation currents (40, 60, 90 μA) under the conditions reported at left. Upper row shows implanted but nonstimulated saline-injected (sham). The sets of traces below show LPS injected and nonstimulated (with LPS only), with LPS + VNS, and with i.p. injection of the nicotinic α_7 receptor PHA543613 (PHA). $\star P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the protective effect of the VNS action, we repeated the same experiment (LPS injection and VNS) after severing the vagus nerve to be stimulated *above* the stimulating cuff, so that the sensory branch of the vagal nerve would not be stimulated (LPS + VNS + VGX). As displayed in the representative couple of traces in Figure 2A (center lower; Fig. 2B, average in blue bar), under these conditions the effect of VNS was similar to the effect without vagotomy (n = 12; n.s., unpaired Student's *t*-test), suggesting that the effect of VNS was determined by activation of the descending (motor) branch. Time course and details of the protocol are given in Materials and Methods.

Because it is known that nicotinic α_7 receptors mediate the anti-inflammatory reflex, we also tried to activate such process directly with i.p. injections of the nicotinic α_7 agonist PHA543613 (2 mg/kg; LPS + PHA). Similar to VNS, the injection of PHA resulted in the block of the LPS-induced reduction in the sI/E ratio, as shown in the representative traces in Figure 2A (lower right; Fig. 2B, average in pink bar, n.s., unpaired Student's *t*-test; n = 5).

VNS and PHA543613 Block the LPS-Induced eIPSC Decrease

Because LPS injection preferentially (or exclusively) appears to target inhibitory vs. excitatory synapses, we sought to determine the effect of the activation of LPS in the absence and in the presence of the anti-inflammatory reflex on eIPSCs in a range of stimulation intensities used to evoke pharmacologically isolated IPSCs. Axon afferents in the brain slice were stimulated with currents in the range up to 120 μA.

We recorded eIPSC in a subset of the conditions reported in the previous paragraph, saline-injected animals with disconnected vagal nerve stimulator (sham), LPS-injected and disconnected vagal nerve stimulator (LPS), or LPS-injected with VNS (LPS + VNS). Representative traces of these recordings are shown in Figure 3A for the stimulation currents indicated above. LPS injections significantly reduced the average eIPSC amplitude (Fig. 3B; saline black, LPS red; $P < 0.05$, unpaired Student's *t*-test; n = 5 for each condition), whereas VNS prevented such effect throughout the stimulation range (Fig. 3B; LPS + VNS green; n = 5). F3

The i.p. administration of PHA543613 also resulted in an even larger eIPSC response throughout the stimulation range (Fig. 3A, bottom row; Fig. 3B, I/O yellow symbols, n.s., unpaired Student's *t*-test; n = 5), suggesting that activation of the anti-inflammatory through direct pharmacological block of nicotinic α_7 receptors is at least as strong a blocker of the central effects of LPS as VNS. Figure 3C summarizes these data in terms of saturation currents, representing the rightmost value of the I/O curves.

DISCUSSION

The peripheral and central nervous systems communicate with a dense, complex, and largely unknown network of biochemical interactions (Hosoi et al., 2002; Müller et al., 2011). Whereas several studies have focused on the ability of the CNS to produce peripheral ailments (Dhabhar, 2014; Jenkins et al., 2014), this study sought to determine whether the peripheral reduction of inflammation mitigates the central effects of LPS-induced systemic stress. We have previously shown that aseptic inflammation as well as other stressors may lead to a decrease in the sI/E ratio in the TC (Garcia-Oscos et al., 2012). Given the pathogenic potential of this process in the etiology of stress-related neuropsychiatric conditions (Maguire, 2014), particularly schizophrenic psychoses (Pietersen et al., 2014a), we sought to identify effective treatments for the relief of the central effects of peripheral inflammation.

The current results show that VNS completely blocks the decrease in sI/E ratio as well as the decrease in

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the maximum obtainable response (I/O curve saturation current) of the inhibitory response induced by peripheral LPS injection. The independence of this result from the integrity of the ascending branch of the stimulated vagal nerve indicates that activation of the efferent branch of the vagal nerve is responsible for the attenuation of the LPS-induced central effect. The vagal nerve possesses multiple peripheral targets, including the spleen and the liver, which carry out important immune/inflammatory functions and have the potential to release mediators of central effects by direct permeation of the blood–brain barrier, by changes in its permeability, or through activation of the ascending branch of the vagal nerve (Hosoi et al., 2002). The effectiveness of the nicotinic α_7 agonist in reproducing the protective action of VNS, both as blockage of the LPS-induced decrease in sI/O ratio and blockage of the depression of the inhibitory currents I/O saturation levels, corroborates the hypothesis that activation of the anti-inflammatory reflex may attenuate the impairment of central inhibition associated with peripheral inflammation (Wang et al., 2003).

A wealth of data supports the notion that stress is a potential trigger for schizophrenic psychoses (Segarra et al., 2012; Misiak et al., 2014) and that inhibitory synaptic transmission is particularly vulnerable to stress (Geuze et al., 2008; Skilbeck et al., 2010; Maguire, 2014). It is well known, for example, that the appearance of several neuropsychiatric symptoms is susceptible to peripheral acute inflammation and/or elevation of immune indicators. The list of stress-sensitive symptoms includes schizophrenic psychoses (Hains and Arnsten, 2008), epilepsy (Allendorfer and Szaflarski, 2014; Allendorfer et al., 2014), depression (Anacker et al., 2013; Weinstein et al., 2014), tinnitus (Minen et al., 2014), anxiety-spectrum disorders such as panic attacks (Ising et al., 2012), posttraumatic stress disorder (Kross et al., 2008), generalized anxiety (Faravelli et al., 2012), and phobias (Krämer et al., 2012). A critical involvement of the central GABAergic system in the expression of these illnesses is further supported by the ample clinical evidence of the effectiveness of GABA enhancers, such as benzodiazepines and barbiturates, in the symptomatic treatment of such conditions (Ketter et al., 1999). The current data suggest pharmacological as well as nonpharmacological alternatives to the use of enhancers of $GABA_AR$ function (which possess a large potential for abuse and even addiction) in the treatment of conditions mentioned above and offer a complementary explanation of the effectiveness of VNS in the symptomatic relief of epilepsy and depression.

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