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Rapid activation of brainstem nuclei following TLR stimulation of the nasal mucosa

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We recently identified a novel neuroimmune mechanism in the nasal mucosa, in which activation of neuronal Toll-like receptor (TLR) 7 results in upregulation of epithelial TLRs, *via* release of substance P. In the present study, we assessed whether intranasal challenge with the TLR7 agonist R-837 additionally activated neurons in the central nervous system. Within one hour, R-837 induced activation of the nucleus of the solitary tract, as well as a small increase in nasal IL-6, but otherwise in the absence of an overt inflammatory response. It is tempting to speculate that it might be a direct interaction of R-837 with trigeminal neurons in order to alert the central nervous system of invading pathogens.

Key words: Toll-like receptor, TLR7, R-837, brain, cFos

INTRODUCTION

The complex interaction between the nervous system and the immune system is a constantly growing field of research. Numerous studies have highlighted the multifaceted interplay between the peripheral nervous system (PNS) and the immune system, where neurotransmitters and neuropeptides can regulate inflammatory mechanisms, and inflammatory mediators can, in turn, alter neuronal activity and sensitivity (Pavlov and Tracey, 2015). The well-established field of neuroimmune communication has additionally outlined the cross-talk between peripheral inflammation and the central nervous system (CNS), a protective strategy in which changes in CNS activity alter behaviour and lead to modulation of the immune system, to advance recovery following infection (D'Mello and Swain, 2017).

Toll-like receptors (TLRs), a conserved family of pathogen recognition receptors, are responsible for the recognition of invading microorganisms, and are crucial to the development of an innate immune response (Kumar et al., 2011). Viruses are primarily recognized via their pathogen-associated molecular patterns (PAMPs) by the evolutionarily conserved family of TLRs, of which, amongst others, TLR7 is repsonsible for viral recognition (Xagorari and Chlichlia, 2008). Systemic activation of TLRs has long been known to lead to CNS activation, through the release of systemic pro-inflammatory mediators, which communicate with the brain (D'Mello and Swain, 2017). Novel studies are now highlighting more direct interplay between TLRs and the nervous system. For example, numerous TLRs have been shown to be expressed on both peripheral and central neurons (Goethals et al., 2010; Drake et al., 2013; Larsson et al., 2018), stimulation of which can both sensitize and activate neurons (Liu et al., 2010). In addition, neuronal mediators have the ability to modulate the activity of TLRs (Goethals et al., 2010; Jiang et al. 2012). Further, TLRs expressed in the CNS have been shown to play a role in the early CNS development, and consequently, the development of psychiatric disorders (Chen et al., 2019; Haddad et al., 2020).

We recently published a study demonstrating that activation of sensory trigeminal neurons by the TLR7

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agonist R-837, led to a rapid release of substance P, and a consequent upregulation of epithelial TLRs (Larsson et al., 2018). This highlighted the role of neuronal TLRs in the protective response against invading pathogens. In the present study, we investigated whether TLRs could similarly engage the CNS to act as a part of this defense mechanism.

METHODS

Animals

Adult male C57BL/6 mice (6-8 weeks) were obtained from Charles River (Sulzfeld, Germany). They were housed in plastic cages with adsorbent bedding in temperature and light-dark cycle (12 h : 12 h) controlled rooms. Food and water were available *ad libitum*. Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. All animal procedures were approved by the regional animal experimental ethical review board at Karolinska Institutet, Stockholm Sweden (ethical permit numbers: N153-11 and N128-13).

Nasal provocation

Mice were intranasally dosed with R-837 (20 µg/20 µl) or PBS under isoflurane anaesthesia. Fifteen min, 30 min, 1 h and 3 h following provocation, animals were terminally anaesthetised with sodium pentobarbital (Apoteket AB, Sweden).

Nasal lavage

Following euthanization, mouse tracheae were exposed, and small incision was made just below the larynx. A 1 ml syringe containing 300 µl NAL buffer (PBS containing 30% proteinase inhibitor cocktail) attached to a 19 G needle was inserted into the trachea towards the nasal cavity and positioned at the epiglottis. Nasal lavage (NAL) buffer was flushed through the nasal cavity and collected. NAL was frozen on dry ice and stored at -80°C.

Immunohistochemistry

Following euthanization, animals were transcardially perfused with heparinised saline followed by 4% freshly made paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed, post-fixed in 4% PFA overnight, transferred to 30% sucrose for 24 h and subsequently embedded in OCT (TissueTek, Sukara Fintek, Göteborg, Sweden) and frozen in isopentane on dry ice.

Serial 40 µm sections from bregma -0.23 mm to bregma -8.15 mm were collected using a cryostat and stained free-floating for cFos. Briefly, sections were quenched for endogenous peroxidase activity and blocked for non-specific binding, using 1% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) and 3% normal goat serum, respectively. Sections were incubated with anti-Fos antibody (1:20000) (PC38, Merck Millipore, Molsheim, France) for 36 h, followed by a biotinylated goat anti-rabbit antibody (1:200) for 2 h and avidin-biotin-peroxidase complex for 2 h. Staining was visualised using nickel-enhanced 3,3' diaminobenzidine. All reagents were purchased from Vector Laboratories (Peterborough, UK) unless otherwise specified.

Image analysis

Images were acquired on an Olympus Provis microscope (Olympus, Hamburg, Germany), connected to an Olympus U-PS camera, using cellSens software (Olympus). Quantification of staining was performed using ImageJ. Image contrast was adjusted to the same value for all images. The area of interest was selected, and a particle count was performed. Particle size and circularity was kept constant and was pre-established according to an average size and circularity of particles from 10 figures. The area encompassing the nucleus of the solitary tract was cut out using the polygon outline tool and pasted into a new 8-bit ImageJ file. Images were thresholded to 165 or 185. Images from control animals were thresholded to the same threshold value as images from treated animals for the same round of staining. Particles were analysed based on size (10-100) and circularity (0.25-1.00).

Measurement of pro-inflammatory cytokines

Levels of IL-1β, IL-6 and TNF-α were measured in nasal lavage using a modified Bio-Plex Pro Mouse Cytokine 23-plex kit on a Luminex Instrument (BioRad), according to the manufacturer's instructions.

Statistical analysis

Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, California). Changes in cFos expression were assessed with a two-way ANOVA followed by a Sidak's multiple comparison post-test

and alterations in inflammatory mediator production were assessed with non-parametric Man-Whitney U test. A p-value of 0.05 or less was considered statistically significant.

RESULTS

cFos expression following R-837 challenge

To determine if stimulation of nasal TLR7 resulted in activation of the CNS, cFos expression was assessed 15 min, 30 min, 1 h and 3 h after R-837 challenge. No cFos expression could be detected 15 or 30 min post-challenge (data not shown). However, an increase in expression was evident in the ipsi- and contralateral sides of the medial and caudal nucleus of the solitary tract (NTS) 1 h after challenge (Fig. 1A-D). Quantification of staining (Fig. 1E) revealed a significant upregulation of staining following R837 challenge in the medial NTS, and a trend towards an increase in the caudal NTS. The expression had diminished by 3 h post-challenge (data not shown).

Levels of pro-inflammatory mediators following R-387 challenge

It is well-established that pro-inflammatory mediators play a vital role in communication between the immune system and brain (D'Mello and Swain, 2017). To determine if R-837 challenge was associated with local production of pro-inflammatory mediators, levels of IL-1β, TNF-α and IL-6 were assessed in NAL following intranasal challenge with R-837. Sixty minutes after challenge with R-837, levels of IL-6 were elevated (Fig. 2), as compared to PBS-controls, reaching significance for IL-6 (Fig. 2B). Comparatively, no differences in IL-1β or TNF were evident (Fig. 2A, C).

DISCUSSION

We recently published a study indicating that activation of TLR7 on trigeminal neurons leads to rapid release of SP, which in turn upregulates TLRs on nearby epithelial cells (Larsson et al., 2018). This interaction between the PNS and TLRs acts to alert and prime the immune system for further pathogen invasion. In the present study we investigated whether the CNS played a parallel role in rapidly alerting and protecting the body from microbial assault.

Intranasal challenge with the TLR7 agonist R-387 led to activation of neurons in the NTS within one hour of challenge. Challenge was associated with elevated levels of nasal IL-6, whereas no change in levels of IL-1β or TNF-α were apparent. The present study is the first to demonstrate that nasal challenge with TLR agonists leads to rapid activation of brainstem neurons.

The capacity for peripheral inflammatory signals to induce changes in the CNS, a process known as immune-to-brain communication, is by far not a novel concept. For many years, it has been known that systemic and local inflammation leads to activation of central neuronal pathways and *de novo* central immune mediator production, as well as fever and behavioural changes, collectively termed "sickness behaviour" (D'Mello and Swain, 2017). These changes are phycological, being necessary to allow the body to appropriately combat infection (Hart, 1988). Communication is known to occur via two main routes: the humoral route involves the interaction of immune medi-

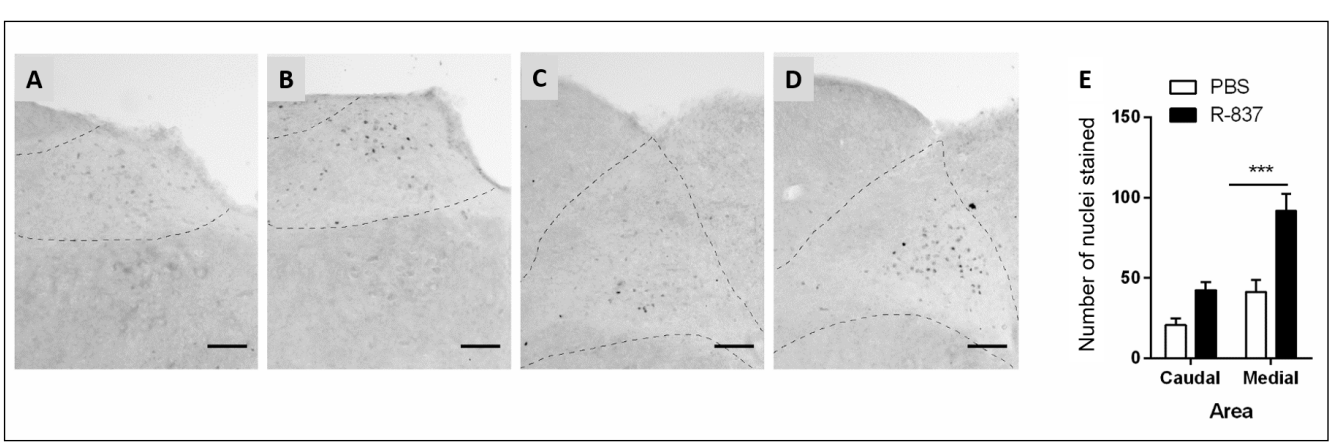

Fig. 1. cFos expression in the nucleus of the solitary (NTS) following intranasal R-837 challenge. Animals were intranasally challenged with PBS (A, C) or R-837 (B, D) and staining was assessed in the caudal (A-B) and medial (C-D) NTS after 1 h. Staining was quantified using image analysis software (E). Values are presented as mean +/- SEM. ***p<0.001 using a two-way ANOVA followed by a Sidak's multiple comparison post-test; n=7.

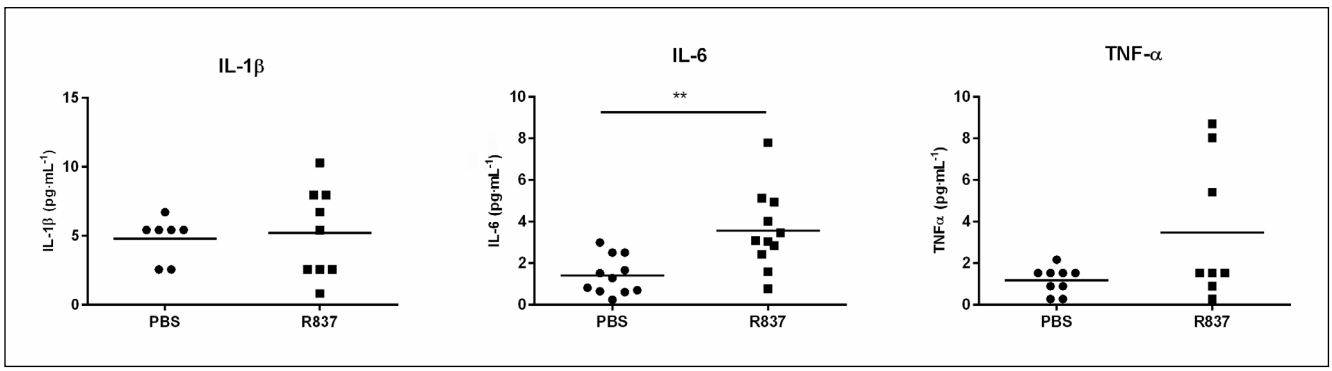

Fig. 2. Levels of pro-inflammatory cytokines in nasal lavage 1 h after intranasal challenge with PBS or R-837. Levels were measured using a multiplex ELISA. Values are presented as individual data points.**p<0.001 using a non-parametric Mann-Whitney U-test; n=8.

ators with cognate receptors at the blood-brain barrier (BBB), as well as direct entry of these mediators to circumventricular organs (CVOs); comparatively, the neuronal route involves interaction of immune mediators with innervating sensory neurons. Numerous studies have indicated that peripheral immune stimulation leads to activation of neurons in many areas and of the brain, as well as behavioural alterations, within 3 h (Sagar et al., 1995). Activation diminishes within 6 h, but changes in neural circuitry and central immune mediator production lead to prolonged alterations in behaviour and mood (Dantzer et al., 2008).

In this study, intranasal challenge with R-837 was associated with a different pattern of CNS activation. Activation was isolated to the brainstem, specifically the NTS, and was evident just one hour after challenge, disappearing within 3 h. Areas related to sickness behaviour (e.g., paraventricular hypothalamus) were unaffected by R-837 challenge. Interestingly, this is in contrast to previous studies that have identified changes in behavior (e.g. fever, decreased motor activity and depression), as well as activation of brain areas such as the area postrema, which were not apparent in this study (Hayasi et al., 2008; Damm et al., 2012; Kubo et al., 2013).

Activation of the CNS following immune stimulation is commonly explained by the production of local and systemic pro-inflammatory cytokines and lipid mediators. Indeed, in previous studies, TLR7-induced overt local and systemic inflammatory responses, leading to CNS activation via both neuronal and humoral routes. Results from the present study indicate that CNS activation following R-837 challenge likely occurs *via* a neuronal route of communication, and the involvement of inflammatory mediators is questionable. Nociceptive affarents from the nasal cavity project directly to the NTS (Anton and Peppel, 1991), suggesting that direct activation of these affarents, may be responsible for the rapid expression of cFos. Indeed the speed of activation, as well as the lack of neuronal activation in surrounding CVOs, such as the area postrema, further discounts the involvement of humoral immune-to-brain communication.

In the present study, nasal mucosal levels of IL-6 were found to be elevated above control levels. However, IL-6 has been shown to sensitize sensory neurons (Andratsch et al., 2009; Durham et al. 2017), but not directly activate them. We and others have previously demonstrated that TLR7 is expressed on neurons in the nasal mucosa and respiratory tract (Drake et al., 2013; Larsson et al., 2018), and that TLR7 agonists can direct activate sensory neurons in culture (Dantzer et al., 2008). This raises the possibility that R-387 directly interacts with neurons in the nasal cavity to induce CNS activation. Indeed, the speed of activation in the CNS and the lack of relevant pro-inflammatory cytokines in the nasal mucosa further corroborate this postulate.

As mentioned above, sensory neurons from the nasal cavity innervate the NTS. Retrograde labelling studies have also demonstrated that these neurons innervate a larger subset of nuclei, including the TBNC and SNC, and that stimulation with nasal irritants results in activation of these areas (Anton and Peppel, 1991) . However, this study showed isolated activation of the NTS. One study has demonstrated that phasic stimulation of nasal nociceptors by CO_2 leads to isolated activation of the NTS (Anton et al., 1991). The authors suggest that the lack of activation of all trigeminal nuclei was related to low-grade stimulation of peripheral affarents, indicating that R-837 may induce low-grade activation of trigeminal neurons.

The role of TLRs in the CNS is not limited to a role in infection and inflammation. Studies have highlighted the TLRs may play a role in early CNS development, particularly in neuronal morphogenesis. Chen et al. (2019) proposed that early activation of TLRs during CNS development may act as an alarm system to facilitate neuronal morphogenesis. Indeed, studies have

shown that maternal TLR3 activation may alter morphogenesis and consequently lead to behavioural and psychiatric disorders in offspring (Haddad et al., 2020). It is clear that TLR activation in the CNS is crucial in many respects, and is worth further study. It is of interest to note that activation of the NTS has been linked to the development of cardiopulmonary reflexes. Due to the convergence of both trigeminal and vagal sensory afferents in this area, it is believed that activation of nociceptive neurons in the nasal cavity can induce vagally-mediated pulmonary reflexes, such as sneeze and apnea (Wallois et al., 1995). This response is protective in nature, acting to eliminate irritating substances from the respiratory tract. The rapid activation of the NTS following R-837 challenge may therefore indicate that the CNS is involved in the immediate protective response to eliminate invading pathogens.

CONCLUSIONS

The acquired data suggest that, in addition to the peripheral nervous system, the central nervous system plays a role in early recognition and possible elimination of viral particles, and that direct activation of nerves by TLR agonists is likely involved in this process.

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