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Full-length Article

Microglial production of TNF-alpha is a key element of sustained fear memory

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ABSTRACT

The proinflammatory cytokine productions in the brain are altered in a process of fear memory formation, indicating a possibility that altered microglial function may contribute to fear memory formation. We aimed to investigate whether and how microglial function contributes to fear memory formation. Expression levels of M1- and M2-type microglial marker molecules in microglia isolated from each conditioned mice group were assessed by real-time PCR and immunohistochemistry. Levels of tumor necrosis factor (TNF)-α, but not of other proinflammatory cytokines produced by M1-type microglia, increased in microglia from mice representing retention of fear memory, and returned to basal levels in microglia from mice representing extinction of fear memory. Administration of inhibitors of TNF-α production facilitated extinction of fear memory. On the other hand, expression levels of M2-type microglia-specific cell adhesion molecules, CD206 and CD209, were decreased in microglia from mice representing retention of fear memory, and returned to basal levels in microglia from mice representing extinction of fear memory. Our findings indicate that microglial TNF-α is a key element of sustained fear memory and suggest that TNF-α inhibitors can be candidate molecules for mitigating posttraumatic reactions caused by persistent fear memory.

1. Introduction

Microglia represent the major population of immune cells of the central nervous system, which produce inflammatory cytokines in response to various environmental stimuli. Microglia are involved in the inflammatory response to harmful substances, such as pathogens, damaged cells, or irritants, and in acute stress response to stimuli such as pain by regulating the production of proinflammatory cytokines. For example, repetitive inescapable tail shocks elevate the levels of the proinflammatory cytokine interleukin (IL)-1β in the hypothalamus of rats (O'Connor et al., 2003), and this response is reversed by the microglial inhibitor minocycline (Blandino et al., 2006, 2009). Moreover, hippocampal microglia isolated from rats exposed to tail-shock stress produce significantly higher levels of IL-1β and the proinflammatory cytokines IL-6 and tumor necrosis factor-α (TNF-α) in response to lipopolysaccharide (LPS) (Frank et al., 2012).

These proinflammatory cytokines are involved in the stress response to electric shock and regulation of contextual fear memory formation caused by electric shock. Contextual fear memory is an associative memory in the context of conditioned fear and arises from stimuli such as an electrical footshock. To generate a long-term contextual fear memory, the unpleasant memory is stabilized through memory consolidation. In contrast, memory extinction induced by continuous or repeated retrieval of fear memory inhibits fear responses. For example, mice with experimental autoimmune encephalomyelitis (EAE) with elevated levels of IL-1β in the hypothalamus exhibit suppressed fear memory consolidation (Acharjee et al., 2013). Furthermore, social isolation induces elevated levels of IL-1β in the hippocampus and cerebral cortex that are associated with suppressed contextual fear conditioning (Pugh et al., 1999).

Administration of IL-6 to the amygdala impairs the acquisition and extinction of conditioned fear in a dose-dependent manner (Hao et al., 2014). LPS-induced elevation of hippocampal levels of

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IL-6 and its downstream transcription factor, signal transducer and activator of transcription 3 (STAT3), which serves as a marker of IL-6 signaling activity, suppresses contextual fear memory consolidation. This suppression is reversed by the inhibition of IL-6 signaling by administering sgp130 (endogenous soluble IL-6 receptor inhibitor) (Burton and Johnson, 2012; Jostock et al., 2001). Intra-amygdala infusion of the proinflammatory cytokine TNF-α delays significantly the acquisition and extinction of auditory fear conditioning (Jing et al., 2014). Collectively, these findings indirectly suggest that the elevation of proinflammatory cytokine levels in the cerebral parenchyma suppress fear memory acquisition, consolidation, and extinction, depending on the affected regions of the brain.

The two subtypes of microglia, M1 and M2, are distinguished by their cell surface markers. M1-type microglial markers include CD16 (Fc gamma III Receptor) and CD32 (Fc gamma II Receptor), and M2-type microglial markers include CD206 (MMR: macrophage mannose receptor) (Kobayashi et al., 2013) and CD209 (DC-SIGN: **d**endritic **c**ell-**s**pecific **i**ntercellular adhesion molecule-3-**g**rabbing **n**on-integrin) (Durafourt et al., 2012). Activated microglia are widely known to exert proinflammatory M1 and anti-inflammatory M2 functions (Boche et al., 2013). M1 cells release proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. In contrast, M2 cells release anti-inflammatory cytokines such as IL-4, IL-5, IL-10, and neurotrophic factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TFG-β), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) (Fumagalli et al., 2011).

All cytokines involved in the acute response to footshock and the suppression of fear memory formation are produced by M1 microglia. Although limited data are available regarding the association between M2 microglial products, such as anti-inflammatory cytokines and growth factors, and the acute response or fear memory, mice lacking microglial BDNF exhibit a significantly decreased freezing fear response to conditioned auditory cues (Parkhurst et al., 2013), indicating that M2 microglial products, including BDNF, may facilitate fear memory formation. These findings suggest that microglial polarization into the M1 and M2 types may be at least partially involved in fear memory formation in an opposite manner; however, this possibility requires investigation.

Here, we aimed to determine whether and how characteristic alterations of microglia are involved in the formation and extinction of fear memory. We show that of the M1-derived proinflammatory cytokines, TNF-α levels increased during retention of fear memory and returned to basal levels during fear memory extinction. Further, the inhibition of TNF-α facilitated the extinction of fear memory. Our findings indicate that microglial TNF-α is a key contributor to sustaining fear memory and that the inhibitors of TNF-α such as minocycline may facilitate extinction of fear memory. Considering that sustained fear memory is a key component of the pathophysiology of posttraumatic stress disorder (PTSD) (Goswami et al., 2013; Yehuda, 2002), inhibition of microglial TNF-α may be a druggable target in developing treatments to alleviate symptoms of PTSD.

2. Materials and methods

2.1. Animals

The animal ethics committee of Tohoku University Graduate School of Medicine approved the experimental protocols. C57BL/6 male mice (SLC Inc., Japan) weighing 20–30 g were individually housed and maintained on a 12:12 light/dark schedule with ad libitum access to food and water throughout the course of the entire experiment.

2.2. Behavioral procedures

Mice were placed into the training chamber (17.5 × 17.5 × 15 cm) with a stainless steel rod floor used to deliver footshocks (Ohara & Co. Ltd.). Each mouse was transferred from its home cage to the training chamber and allowed to explore it for 148 s before receiving a single footshock (2 s, 0.4 mA) following a standardized protocol (Suzuki et al., 2004). After the footshock, mice were left in the training chamber for 30 s and then transferred to their home cage. Twenty-four hours after conditioning, mice were re-exposed to the training chamber for varying durations as follows: 0 min (FS0; footshocked mice with no reexposure), 3 min (FS3; footshocked mice with 3 min of reexposure) or 30 min (FS30; footshocked mice with 30 min of reexposure) without receiving a footshock, and then transferred to their home cage. No (0 min) or short-duration (3 min) reexposure to the context are supposed to result in retention of fear memory, while long-duration (30 min) reexposure is supposed to facilitate extinction of fear memory.

To validate the effect of no, short- or long-duration reexposure on retention or extinction of fear memory, the percentage of time for which the mice exhibited freezing behavior was measured every 5 min during the 30 min exposure session (Mamiya et al., 2009; Suzuki et al., 2004). Control animals were similarly trained without receiving an electric footshock and were re-exposed to the context for 0 min (Con0), 3 min (Con3) or 30 min (Con30). To evaluate their behavior, mice were again transferred to the training chamber 24 h after the reexposure, and the percentage of time the mice engaged in freezing behavior during a 5 min observation (freezing time) was measured as an indicator of the behavioral outcome of fear memory. For analyses of microglial cells, mice were trained, re-exposed to the context in the same manner as the mice in the behavioral evaluation studies, and decapitated 24 h after the reexposure. This procedure was performed instead of behavioral monitoring to avoid the effect of tertiary exposure to the training chamber on the molecular phenotype of microglial cells.

2.3. Isolation of microglia

After the mice were decapitated, the brain was prepared as a single-cell suspension using a neural tissue dissociation kit and a gentleMACS Dissociator (Miltenyi Biotec Bergisch Gladbach, Germany). CD11b-positive microglia were isolated using CD11b-labeled MicroBeads and a magnetic cell separator (Miltenyi Biotec) (Yu et al., 2015). To verify the purity of the isolated microglia samples, cells separated with CD11b-labeled MicroBeads were stained by incubating with rat anti-mouse CD11b FITC-conjugated monoclonal antibodies (mAb) (Miltenyi Biotec) for 5 min at 4 °C. After the mAb-stained subjects were washed, cells were subjected to flow cytometry with an ACCURI Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI, USA). Purities of CD11b-positive cells were above 97% for all subjects (Suppl. Fig. 1).

2.4. Quantitative real-time PCR

Total RNA was extracted from CD11b-positive microglia and used as the template for cDNA synthesis conducted using random primers and the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). The relative copy number of each transcript in each cDNA sample was measured using specific primers and the iQ SYBR Green Supermix (Bio-Rad Inc., Hercules, CA, USA). Representative cell surface markers, cytokines, and growth factors specifically produced by M1 or M2 phenotypes of microglia were