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Brain inflammation induces post-synaptic changes during early synapse formation in adult-born hippocampal neurons ☆

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ABSTRACT

An inflammatory reaction in the brain is primarily characterized by activation of parenchymal microglial cells. Microglia regulate several aspects of adult neurogenesis, i.e. the continuous production of new neurons in the adult brain. Hippocampal neurogenesis is thought to be important for memory formation, but its role in brain diseases is not clear. We have previously shown that brain inflammation modulates the functional integration of newly formed hippocampal neurons. Here, we explored whether there is a defined time period during synaptic development when new neurons are susceptible to brain inflammation. Newly formed hippocampal neurons, born in an intact environment in the adult mouse brain, were exposed to lipopolysaccharide (LPS)-induced inflammation during either early or late phases of excitatory and inhibitory synaptogenesis. We used intra-hippocampal injections of GFP-retroviral vector (RV-GFP) to label the new neurons and ipsilateral LPS injection at either 1 or 4 weeks post-RV-GFP injection. A single intra-hippocampal LPS injection induced an inflammatory response for at least 3 weeks, including an acute transient pro-inflammatory cytokine release as well as a sub-acute and sustained change in microglial morphology. The general cytoarchitecture of the hippocampal dentate gyrus, including granule cell layer (GCL) volume, and astrocytic glial fibrillary acidic protein expression was not different compared to vehicle controls, and no Fluoro-Jade-positive cell death was observed. New neurons encountering this inflammatory environment exhibited no changes in their gross morphology. However, when inflammation occurred during early stages of synapse formation, we found a region-specific increase in the number of thin dendritic spines and post-synaptic density-95 (PSD-95) cluster formation on spines, suggesting an enhanced excitatory synaptic connectivity in the newborn neurons. No changes were observed in the expression of N-cadherin, an adhesion molecule primarily associated with excitatory synapses. At the inhibitory synapses, alterations due to inflammation were also evident during early but not later stages of synaptic development. Gephyrin, an inhibitory scaffolding protein, was down-regulated in the somatic region, while the adhesion molecules neuroligin-2 (NL-2) and neurofascin were increased in the somatic region and/or on the dendrites. The $GABA_A$ receptor-α2 subunit ($GABA_AR$-α2) was increased, while pre/peri-synaptic GABA clustering remained unaltered. The disproportional changes in post-synaptic adhesion molecules and $GABA_A$ receptor compared to scaffolding protein expression at the inhibitory synapses during brain inflammation are likely to cause an imbalance in GABAergic transmission. These changes were specific for the newborn neurons and were not observed when estimating the overall expression of gephyrin, NL-2, and $GABA_AR$-α2 in the hippocampal GCL. The expression of interleukin-1-type 1 receptor (IL-1R1) on preferentially the somatic region of new neurons, often in close apposition to NL-2 clusters, may indicate a direct interaction between brain inflammation and synaptic proteins on newborn neurons. In summary, this study provides evidence that adult-born hippocampal neurons alter their inhibitory and excitatory synaptic integration when encountering an LPS-induced brain inflammation during the initial stages of synapse formation. Changes at this critical developmental period are likely to interfere with the physiological functions of new neurons within the hippocampus.

Introduction

The adult mammalian brain is endowed with the capacity to produce new neurons throughout the lifetime of an organism. This phenomenon of adult neurogenesis is restricted to two brain regions; the subventricular zone lining the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Curtis et al., 2007; Eriksson et al., 1998; Zhao et al., 2008). The newly formed neurons integrate into the adult

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neuronal circuitry of the olfactory bulb and hippocampus as interneurons and granule cells, respectively. In humans, the annual turnover of hippocampal granule cells has been estimated to be 1.75% (Spalding et al., 2013) and according to animal studies they may contribute to memory formation (Gu et al., 2012; Kee et al., 2007). However, their role during brain diseases is not clear. Various pathological insults, including epileptic seizures and stroke, modulate different aspects of adult neurogenesis, ranging from cell proliferation, migration, differentiation, survival, and subsequently functional integration (Arvidsson et al., 2002; Bengzon et al., 1997; Ekdahl et al., 2001; Jakubs et al., 2006; Parent et al., 1997). One of the hallmarks of these brain pathologies is neuroinflammation, which primarily involves innate immune signaling through activation of parenchymal microglial cells (Danton and Dietrich 2003; Gorter et al., 2006), but also an adaptive immune response with the recruitment of systemic immune cells (Mosley et al., 2012; Shichita et al., 2009; Wraith and Nicholson 2012).

Lipopolysaccharide (LPS) is an endotoxin of gram-negative bacteria, used extensively for inducing an immune response in the brain both through peripheral (Laflamme et al., 2003) and intra-cerebral administration (Deng et al., 2012; Herber et al., 2006; Zhou et al., 2012). LPS-induced microglial activation in the brain can be characterized by an increase in the proliferation and migration of the cells, as well as changes in their expression/secretion of inflammatory mediators. However, several studies have stressed the dual role of activation, by which the microglia may exert both detrimental/neurodamaging and beneficial/neuroprotective effects, possibly through differential expression of pro- (i.e. interleukin (IL) -1β, IL-6 or tumor necrosis factor (TNF)-α) and anti- (i.e. IL-4, IL-10 or transforming growth factor (TGF)-β) inflammatory cytokines, respectively (Nguyen et al., 2002). Consistently, there is accumulating evidence suggesting that microglial activation may simultaneously or sequentially both promote and prohibit adult neurogenesis, depending on the activation stage and the functional balance of secreted molecules with pro- and anti-inflammatory actions (Butovsky et al., 2006; Ekdahl et al., 2003; Ekdahl et al., 2009; Monje et al., 2003). Most of these studies have focused on the initial stages of neurogenesis including proliferation, survival and neuronal fate determination. The role of microglia at later stages, i.e., during synaptic assembly, stability, and transmission, is just beginning to be unraveled (Ekdahl 2012).

Adhesion molecules are one of the potential candidates linking the microglial cells to the synaptic integration of adult-born neurons. They are crucial not only during synaptic assembly and development but also in fine-tuning the synaptic response (Arikkath and Reichardt 2008; Dalva et al., 2007; Washbourne et al., 2004). We have previously shown that the expression of neuroligin-2 (NL-2), an adhesion molecule primarily associated with inhibitory synapses, together with the post-synaptic scaffolding protein gephyrin is significantly altered on newly formed hippocampal neurons in a model of partial *status epilepticus* (SE) (Jackson et al., 2012). Interestingly, inflammatory mediators are suggested to regulate the expression of adhesion molecules either directly or indirectly. In fact, N-cadherin, an adhesion molecule present at excitatory synapses is modulated by TNF-α (Kubota et al., 2009). Additionally, IL-1 receptor accessory protein (IL-1RAcP), an essential component mediating IL-1 cytokine-related immune responses, can itself act as a trans-synaptic cell adhesion molecule in neurons and organize synapse formation (Yoshida et al., 2012). These studies indicate that adhesion molecules may function as one of the important players in potential signaling pathways between microglia and neurons.

The objectives of the present study were, first, to describe the temporal development of the inflammatory response in the hippocampal dentate gyrus following a single intra-hippocampal LPS injection and, secondly, to analyze the expression of synaptic adhesion molecules and scaffolding proteins related to excitatory and inhibitory transmission on newly formed hippocampal neurons encountering the LPS-induced inflammation during either early or late phase of synaptic development.

Materials and methods

Animals and group assignment

In total, ninety-eight adult male C57BL/6 mice (Charles Rivers), weighing 25 g each at the beginning of the experiments, were used. The mice were housed under a constant 12 h light/dark cycle with access to food and water ad libitum. All experimental procedures followed the guidelines set by the Malmö-Lund Ethical Committee for the use and care of laboratory animals.

The animals were included in the following three sets of experiments: Experiment 1: Fifteen mice were used to optimize the intra-hippocampal LPS injections in order to achieve sustained microglial activation throughout the unilateral hippocampal dentate gyrus. This included two different doses of LPS (0.5 μl of 0.5 or 1 μg/μl) and two different stereotaxic coordinates at 2 survival time points (3 days and 3 weeks). Experiment 2: Eighty-three mice were used for histological ($n = 47$, including mice used in Experiment 3) and biochemical ($n = 36$) characterization of LPS-induced inflammation in the ipsilateral hippocampal dentate gyrus. The mice received a single intra-hippocampal injection of LPS or vehicle and were then perfused at three different time points (12 h, 3 days and 3 weeks). Experiment 3: Thirty-three mice were used for evaluation of synaptic protein expression on newly formed hippocampal neurons following intra-hippocampal LPS injections. These mice first received a stereotaxic injection of green fluorescent protein-expressing retroviral vector (RV-GFP) in the hippocampal dentate gyrus. One or four weeks later, LPS or vehicle was injected into the ipsilateral hippocampus. The mice were then perfused 3 weeks later (at 28 days post-injection (dpi) of RV-GFP and 49 dpi, respectively) for immunohistochemical and confocal analyses.

Lipopolysaccharide (LPS) administration

Animals were anesthetized with isofluorane (1%) and LPS from *Salmonella enterica*, serotype *abortus equi* (Sigma-Aldrich, Sweden; 0.5 μg in 0.5 μl of artificial CSF (aCSF)) or vehicle (0.5 μl of aCSF) was stereotaxically injected into the left dorsal hippocampus (coordinates: 3.0 mm caudal and 2.5 mm lateral to bregma, and 1.9 mm ventral to dura; toothbar set at −2.2 mm) (Paxinos and Watson 1997) using a glass microcapillary.

Labeling of new neurons

Animals were anesthetized with isofluorane (1%) and were stereotaxically injected with 1.5 μl of retroviral vector, containing the GFP gene under the CAG promoter ($4.8 * 10^8$ TU/ml), unilaterally into the left dorsal hippocampus (coordinates: 2.0 mm caudal and 1.5 mm lateral to bregma, and 1.8 mm ventral to dura; toothbar set at −2.2 mm) using a glass microcapillary (similar to (Laplagne et al., 2006)).

Enzyme-linked immunosorbent assay (ELISA)

At 12 h, 3 days and 3 weeks after intra-hippocampal LPS or vehicle injection, mice received an overdose of sodium pentobarbital (200 mg/kg, i.p.) and were trans-cardially perfused with ice-cold saline and whole hippocampus, ipsilateral to the LPS or vehicle injection, was rapidly removed and quickly frozen on dry ice. Samples were homogenized on ice in buffer (pH 7.6), containing (in mM): 50.0 Tris–HCl, 150 NaCl, 5.0 $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100, and then centrifuged at 17,000 times gravity for 30 min at 4 °C. Protein concentration was determined in supernatants by BCA protein assay as per manufacturer's instructions (Pierce, USA). IL-1β, IL-6, IL-4, and IL-10 concentrations were determined by ELISA (Duoset; R & D Systems, USA) according to the manufacturer's protocol.