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Novel connection between newborn granule neurons and the hippocampal CA2 field

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ABSTRACT

Newborn neurons are continuously added to the hippocampal dentate gyrus (DG) throughout life. Mature and immature granule neurons are believed to send their axonal projections exclusively to the hippocampal CA3 field. However, recent data point to an alternative trisynaptic circuit, involving a direct axonal projection from mature granule neurons to the CA2 field. Whether this circuit takes place only in mature granule neurons or, on the contrary, whether immature granule neurons also contribute to this novel connection is unknown. We used various retroviral vectors to show that immature granule neurons send axonal processes to and establish synaptic contacts with CA2 pyramidal neurons and that axonal growth follows a similar time course to that described for CA3 innervation. In addition, we provide experimental evidence demonstrating that the pathway connecting newborn granule neurons and the CA2 field can be modulated by physiological and deleterious stimuli.

Introduction

Newborn neurons are continuously added to the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) throughout life. Progenitor cells asymmetrically divide and give rise to transiently amplifying neuronal precursors. Immature neuroblasts actively proliferate and differentiate into mature granule neurons, going through various stages of maturation. During the differentiation of newborn neurons their dendritic tree complexity increases, and axons are projected towards the CA3 region (Zhao et al., 2006), establishing functional synapses with CA3 pyramidal neurons (Toni et al., 2008). At the end of this maturation process, newborn neurons are fully integrated in the classic trisynaptic circuit and are functionally indistinguishable from surrounding mature granule neurons (Zhao et al., 2006). However, during immaturity, newborn neurons are characterized by a lower activation threshold (Schmidt-Hieber et al., 2004), and adult hippocampal neurogenesis (AHN) is involved in hippocampal-dependent learning, being crucial for pattern separation (Clelland et al., 2009; Nakashiba et al., 2012). AHN is a tightly regulated process modulated by numerous external stimuli.

It has been widely demonstrated that the classic hippocampal trisynaptic circuit (Enthorrinal Cortex ((EC))-DG-CA3-CA1) is one of the most important networks involved in memory and learning processes. However, recent data point to the existence of an alternative trisynaptic circuit (Kohara et al., 2014) that involves the connection between mature granule neurons and CA2 pyramidal neurons. This alternative circuit is crucial for contextual and social memory (Hitti and Siegelbaum, 2014; Wintzer et al., 2014). However, whether newborn granule neurons contribute to this connection remains unknown. Here we analyzed the potential contribution and time course of the connections between newborn granule neurons and the hippocampal CA2 region. To this end, we used various retroviral vectors (encoding for either GFP or Synaptophysin-GFP), and specific markers for the axons of newborn neurons (3R-Tau) and for the CA2 field (RGS14 and PCP4) (Antonio et al., 2014; Evans et al., 2014). In addition, we show how both deleterious and neuroprotective stimuli modulate these connections.

Material and methods

Animals

Six-week-old female C57BL/6JRcc mice were obtained from Harlan Laboratories. Animals were housed in a specific pathogen-free colony facility, in accordance with European Community Guidelines (directive 86/609/EEC), and handled following European and local animal care protocols. Animal experiments received the approval of the CBMSO's Ethics Committee. Five mice per experimental condition and time point were used in all the experiments.

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Physical exercise protocol

Animals were habituated to the treadmill apparatus for 15 min (Cibertec, Madrid, Spain). The running speed was 0.2 m/s. Animals ran 7 days per week for 40 min/day over 2 weeks. Sedentary animals remained on the treadmill without running for the same period of time. Animals were eight weeks old at the beginning of the exercise protocol.

Treatment with lipopolysaccharide from Escherichia coli (LPS)

LPS was chronically administered during 8 weeks subcutaneously via Alzet® osmotic pumps (model 1004) (Durect Corporation, Cupertino, California). To obtain a continuous LPS delivery of 300 μg/kg/day for 8 weeks, pumps were filled with a solution of LPS (Sigma, from *E. coli* 055:B5, St. Louis, MO) diluted in 0.1 M PBS. Pumps were replaced by new ones 4 weeks after the beginning of the treatment. Control mice carried a PBS-containing osmotic pump and were subjected to the same experimental manipulations than LPS-treated mice. Animals were eight weeks old when they were operated for the first time.

Retroviral stock preparation

We used two retroviral stocks, which varied in the genes encoded: CAG-GFP encoding for GFP (Zhao et al., 2006) and Synaptophysin-GFP (Syn-GFP) for GFP fused with Synaptophysin (Kelsch et al., 2009). Since both retroviruses used are engineered to be replication incompetent, only dividing cells at the time of surgery can be infected (Kelsch et al., 2008; Zhao et al., 2006). On the one hand, GFP-expressing retroviruses were used to allow the visualization of the whole morphology (including the MFT) of the newborn neurons. On the other hand, the Syn-GFP retrovirus allowed Syn^+ presynaptic cluster visualization. Moreover, anti-GFP immunohistochemistry allowed axon and whole mossy fiber presynaptic terminal visualization when using this type of retrovirus (Kelsch et al., 2009). The plasmids used for the production of the GFP-expressing retrovirus were a generous gift from Prof. Fred H. Gage (Salk Institute), while those used to produce the Syn-GFP retrovirus were kindly provided by Prof. Carlos Lois (University of Massachusetts). Retroviral stocks were concentrated to working titers of 1×10^7 pfu/ml by ultracentrifugation (Zhao et al., 2006).

The time course of newborn neuron-CA2 synaptic contact establishment was studied by using the GFP-expressing retrovirus, whereas the modulation by LPS was analyzed by using the Syn-GFP retrovirus. Although the presence of Synaptophysin at the synaptic bouton is not a measure of functionality, it further confirms their existence and possibility of function.

Stereotaxic surgery

Mice were anesthetized with Isoflouran and placed in a stereotaxic frame. Coordinates (mm) relative to the bregma in the anteroposterior, mediolateral, and dorsoventral planes were as follows: DG [−2.0, 1.4, 2.2]. 2 μl/DG of virus solution was infused at 0.2 μl/min via a glass micropipette. Animals were 8 weeks old at the moment of retroviral injections. Five mice per experimental condition and time point were used for retroviral labeling experiments.

Sacrifice

Mice were fully anesthetized by means of an intraperitoneal injection of pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffer. Brains were removed and post-fixed overnight in the same fixative.

Immunohistochemistry

Sagittal brain sections were obtained on a Leica VT1200S vibratome (50-μm thick sections). Immunohistochemistry was performed as described previously (Llorens-Martin et al., 2013). For all the immunohistochemical analysis, series of brain slices were made up randomly of one section from every 9th. One series, containing 8–10 sections, was used for each immunohistochemical determination. The following primary antibodies were used: rabbit anti-GFP (Molecular Probes (Invitrogen), 1:1000); mouse anti-RGS14 (Neuromab, 1: 1,000); mouse anti-3RTau (EMD Millipore, 1:500); and rabbit anti-PCP4 (Sigma-Aldrich, 1:500). The following secondary Alexa-conjugated antibodies were used at a final concentration of 1:1,000: Donkey Alexa 555-conjugated anti-rabbit (Life Technologies); Donkey Alexa 488-conjugated anti-mouse (Life Technologies); Donkey Alexa 488-conjugated anti-rabbit, (Life Technologies); Donkey Alexa 647-conjugated anti-rabbit (Life Technologies). All sections were counterstained with DAPI (Calbiochem, 1:5000). In the case of 3RTau immunohistochemistry, an antigen retrieval step was required to visualize the axons of newborn neurons (Llorens-Martin et al., 2012).

Mossy fiber terminal area measurement

Ten stacks of images per mouse and anatomical region were obtained in a LSM710 Zeiss confocal microscope. Stacks of images were randomly obtained among the different sections composing the series (63× Oil immersion objective, xy dimensions: 60 μm) (Z-axis interval: 0.13 μm). The area of each individual mossy fiber terminal (MFT) contained in the images was measured in both the CA2 and CA3 regions. Data averaged on the five mice are presented in the graphs and have been used to perform statistics. A minimum of 500 terminals per experimental condition and time point were measured. GFP Z-projections were obtained and the area of each terminal bouton was measured manually in ImageJ as previously described (Zhao et al., 2006). All the different measurements and analysis were performed by the same investigator blinded to experimental condition.

$Synaptophysin^+$ area of the mossy fiber terminal quantification

Ten stacks of images per mouse and anatomical region were obtained in a LSM710 Zeiss confocal microscope. Stacks of images were randomly obtained among the different sections composing the series. The area of

Fig. 1. Time course of DG-CA2 innervation. Representative images showing newborn granule neurons at 1 (A–E), 2 (F–J), 3 (K–O), 4 (P–T), and 8 (U–Y) weeks of age labeled with a GFP-expressing retrovirus (green). A, F, K, P and U represent Tile Scan acquisitions (25× Oil Objective) showing the whole anatomy of the hippocampus. B, G, L, Q and V show 25× magnifications of the CA2 region. In C, H, M, R and W, 25× magnifications of the CA3 region can be observed. D, I, N, S and X represent a 25× magnification of the hilar region. Finally, E, J, O, T and Y show high power magnifications (63× oil objective) of the CA2 region. One week after retroviral injection, no mossy fiber terminals (MFTs) can be observed in the CA2 field (E). In 2-week-old cells, mossy fibers are observable in the CA2 region (J), although no MFTs are detected. In cells 3 weeks old (O) and onwards (T, Y and AD), small MFTs are observed in the dendritic shaft and proximal domain of CA2 dendrites in the *stratum lucidum*. Z: Quantification of the MFT area at different cell ages and in the different hippocampal subfields. MFT area increased with neuron age in both CA2 and CA3 regions, although CA2 MFTs were smaller than CA3 MFT at all the cell ages studied. AA: Frequency histogram showing the distribution of MFT sizes at the different cell ages and hippocampal subfields. It can be observed how, at younger cell ages (3 weeks post-injection), most of CA2 and CA3 MFT are smaller than 1 μm^2. In contrast, an increased proportion of MFT displayed larger areas 8 weeks post-injection. DG: dentate gyrus. H: hilus. SO: *stratum oriens*. SP: *stratum pyramidale*. SL: *stratum lucidum*. SR: *stratum radiatum*. SLM: *stratum lacunosum moleculare*. MFT: mossy fiber terminal. White triangles indicate the presence of a MFT. White scale bar: 200 μm. Yellow scale bar: 50 μm. Green scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)