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Congenital lack of nNOS impairs long-term social recognition memory and alters the olfactory bulb proteome

Mathias Jüch [a], Karl-Heinz Smalla [b], Thilo Kähne [c], Gert Lubec [d], Wolfgang Tischmeyer [b], Eckart D. Gundelfinger [b], Mario Engelmann [a,*]

[a] Otto-von-Guericke-Universität, Institut für Biochemie und Zellbiologie, Leipziger Straße 44, D-39120, Magdeburg, Germany
[b] Leibniz-Institut für Neurobiologie, Brenneckestrasse 6 D-39118 Magdeburg, Germany
[c] Otto-von-Guericke-Universität, Institut für Experimentelle Innere Medizin, Leipziger Straße 44, D-39120 Magdeburg, Germany
[d] Medizinische Universität Wien, Klinische Abteilung für Allgemeine Pädiatrie und Neonatologie, Währinger Gürtel 18, A-1090 Wien, Austria

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ABSTRACT

The gaseous neurotransmitter nitric oxide (NO), synthesized by the enzyme neuronal nitric oxide synthase (nNOS), is thought to play a major role in the modulation of memory. We tested adult nNOS-deficient and wild-type mice for their recognition memory abilities in the social discrimination paradigm, which is based on olfactory cues. Subsequently, proteomic investigation of the olfactory bulbs of both genotypes were performed under basal conditions and 6 h after learning, i.e., during the consolidation of long-term memory. Short-term and intermediate-term recognition memory was normal in nNOS-deficient mice. However, unlike wild-type mice, nNOS-deficient mice failed to consolidate an olfactory cued long-term recognition memory. Proteomic analysis revealed changes in glycolytic enzymes (e.g., fructose-bisphosphate aldolase C, glyceraldehyde-3-phosphate dehydrogenase), voltage-dependent anion-selective channels 1 and 2, alpha-synuclein, F-actin-interacting proteins (e.g., neuronal protein 25/transgelin 3), proteins of the ubiquitin proteasome system, and heterogeneous nuclear ribonucleoproteins implicated in the regulation of messenger RNA trafficking, stability and translation. Our data suggest that, in the mouse, NO of nNOS origin is critically involved in the regulation of protein synthesis-dependent olfactory long-term memory consolidation within relevant brain structures including the olfactory bulb.
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1. Introduction

There is a vast literature suggesting that memory consolidation, i.e., the conversion of newly acquired memory to a more permanent state, is linked to *de novo* protein synthesis in relevant brain structures (Davis & Squire, 1984; Matthies, 1989; McGaugh, 2000; Stork & Welzl, 1999). Signalling pathways that control the translation of mRNAs are therefore likely to regulate the synaptic plasticity that underlies long-term memory.

Nitric oxide (NO) of neuronal NO synthase (nNOS) origin is a retrograde neuromessenger that facilitates long-term potentiation (Jacoby, Sims, & Hartell, 2001; Malen & Chapman, 1997), an electrophysiological phenomenon of synaptic plasticity thought to be associated with learning and memory processes, and there is evidence that it supports long-term memory formation (Hölscher, McGlinchey, Anwyl, & Rowan, 1996; Hölscher & Rose, 1992). For example, nNOS-deficient mice perform worse than wild-type controls in the Morris water maze (Kirchner et al., 2004), and show a rapid extinction of long-term place preference memory (Itzhak & Anderson, 2007). In non-neuronal cells, NO targets the translational machinery via signaling pathways involving extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) (Pervin, Singh, Hernandez, Wu, & Chaudhuri, 2007). In the nervous system, these kinases are implicated in the regulation of translation during long-term potentiation and memory consolidation (Jaworski & Sheng, 2006; Kelleher, Govindarajan, & Tonegawa, 2004).

Recognition memory refers to the ability to discriminate between familiar and unfamiliar stimuli. Among the different laboratory tasks used to investigate recognition memory in mice, the social recognition/social discrimination paradigm has proved particularly advantageous. This task relies on spontaneous investigatory behavior, as mice have an innate drive to investigate unfamiliar over familiar conspecifics. Thus this task does not require additional stimuli to provoke the behavioral response used as readout for the memory (Dantzer, Bluthé, Koob, & Le Moal, 1987; Engelmann, Wotjak, & Landgraf, 1995), avoiding the

* Corresponding author. Address: Otto-von-Guericke-Universität, AG Neuroendokrinologie and Verhalten, Institut für Biochemie und Zellbiologie, Leipziger Str. 44/Haus 36, D-39120 Magdeburg, Germany. Fax: +49 391 67 14365.
E-mail address: mario.engelmann@med.ovgu.de (M. Engelmann).

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problem of interpreting data from behavioral tests in which conditioned and unconditioned stimuli are applied. To solve the task, mice have to acquire and process the conspecific's individual odour bouquets (''olfactory signatures'') which comprise both volatile and non-volatile fractions (Richter, Wolf, & Engelmann, 2005; Rock, Mueller, Weimar, Rammensee, & Overath, 2006). They do this by sniffing and licking at the conspecific, and subsequently process the olfactory stimuli at the level of the olfactory bulb.

Adult male mice of the C57BL/6 strain can store the information of an individual olfactory signature of a conspecific for at least 24 h (Kogan, Frankland, & Silva, 2000). In the social recognition/social discrimination paradigm, the formation of long-term ($\geqslant$24 h) memory requires two stages of protein synthesis (Richter et al., 2005; Wanisch, Wotjak, & Engelmann, 2008). The first stage is essential for an intermediate-term maintenance of memory for at least 5 h (Engelmann, 2009). It starts during or shortly after learning and involves the induction c-Fos, a transcription factor constituent implicated in memory consolidation. (Guzowski, 2002; Tischmeyer & Grimm, 1999) in cells of the main and accessory olfactory bulbs and associated brain areas approximately 1 h after olfactory recognition learning (Richter et al., 2005). The second stage of protein synthesis-dependent memory consolidation starts about 6 h after learning. It depends upon the undisturbed passage of the first stage (Richter et al., 2005; Wanisch et al., 2008), implying that – at the olfactory bulb – gene expression-dependent changes during the early stage might participate in the control of the late stage of memory consolidation.

These findings suggest that olfactory recognition memory requires the proper regulation of learning-induced sequential translational changes at the level of the olfactory bulb. Because in mice this brain area shows a remarkably high amount of nNOS (Breer, Klemm, & Boekhoff, 1992; Kishimoto, Keverne, Hardwick, & Emson, 1993; Kosaka & Kosaka, 2007), the present study was designed to test whether nNOS is important for olfactory recognition learning and memory formation.

We report here that nNOS-deficient mice show a normal social discrimination 1 h, 3 h, and 6 h after learning, indicating an intact memory acquisition, short-term memory retention, and even intermediate-term memory retention. However, unlike wild-type mice, nNOS-deficient mice failed to retain the memory for 24 h. This suggests an impaired ability to form long-term memory, which requires not only an early stage, but also a late stage of protein synthesis.

We analyzed the consequences of the congenital lack of nNOS on the proteomic composition of the olfactory bulb. We assessed proteomic state differences both under basal conditions and during consolidation of the acquired olfactory recognition memory at 6 h after learning, a time point that marks the beginning of the late phase of translation-dependent memory consolidation (Richter et al., 2005; Wanisch et al., 2008). The proteomic state at this stage of memory processing might be particularly important for the ability to stabilize the engram and, hence, for the behavioral responses of the mice when monitored 24 h after learning.

2. Material and methods

2.1. Animals

Male mice with a targeted disruption of the nNOS gene (nNOS-deficient) showing >95% loss of NO production in the central nervous system (Huang, Dawson, Bredt, Snyder, & Fishman, 1993) and wild-type mice of the C57BL/6 J strain, which share >99.9% genetic similarity with the nNOS-deficient mice (Gammie & Nelson, 1999), obtained from the same source (Prof. P. L. Huang, Massachusetts General Hospital, Boston, USA) were used. The genetic background is a combination of the 129Xl/SvJ and C57BL/6 J strains with a predominance of C57BL/6 J, because mice were backcrossed for three generations into C57BL/6 J and then intercrossed to obtain nNOS-deficient mice and wild-type littermates, which were subsequently bred in separate lines. In the olfactory recognition paradigm used in the present study (see below), mice of the 129SV strain, the C57BL/6 J and the C57BL/6JOlaHsd strains, as well as mice obtained from a 129SV $\times$ C57BL/6JOlaHsd heterozygous breeding showed an intact ability to form a long-term recognition memory (unpublished data), implying that strain-specific differences do not have an impact on the memory performance in the mice tested in this study.

Animals (25–36 weeks old) were housed in groups under standard laboratory conditions with a 12 h light/12 h dark cycle (light on 7:00 a.m.) for 1 week before starting the experiments. Food and water were available *ad libitum*. Juvenile mice of both sexes (28–39 days old) were used as olfactory stimuli. All experimental manipulations were approved by the Committee on Animal Health and Care of the local governmental body and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals (86/609/CEE).

2.2. Olfactory recognition memory

Behavioral experiments were started during the beginning of the light phase, i.e., between 8:00 and 12:00 a.m. Olfactory recognition of 20 wild-type and 19 nNOS-deficient mice was tested using the social discrimination procedure adapted from rat studies (Engelmann et al., 1995). Briefly, experimental subjects were separated by transferring them to fresh small cages (size 14 $\times$ 20 $\times$ 15 cm) 2 h before starting the session. A social discrimination session consisted of two 4-min exposures of a given juvenile to an adult in the adult's cage. During the first encounter (''learning''), the duration of investigatory behavior of the adult towards the juvenile (mainly sniffing and licking of the anogenital region of the juvenile) was recorded by a trained observer with the help of software running on a personal computer. The juvenile was then removed and kept individually in a fresh cage with food and water *ad libitum*. After a defined retention interval of either 1 h, 3 h, 6 h, or 24 h, the juvenile was re-exposed to the adult (second exposure; ''choice''), but this time together with an additional, previously not presented juvenile of the same mouse strain. The duration of investigatory behavior of the adult towards each juvenile was measured separately. Investigating the new juvenile significantly longer than the sampled juvenile (i.e., the conspecific previously presented during learning) during choice was taken as evidence for an intact recognition memory (Engelmann et al., 1995). In parallel to the investigation duration, the trained observer monitored also the duration the experimental subjects spent in aggressive (e.g., biting, chasing, and threatening the juvenile) and sexual behavior (e.g., attempts to copulate) during learning in experiments with 24 h retention interval.

For the proteome analysis (see below) under basal conditions (i.e., non-exposed) wild-type and nNOS-deficient mice (n = 8 per group) were separated before being sacrificed under resting conditions by quick decapitation. Additional five wild-type and five nNOS-deficient mice were exposed to a juvenile as described above for learning and killed 6 h later. Immediately after decapitation, the brains were removed from the scull in an ambient temperature of -20 °C and the olfactory bulbs (containing both the main olfactory bulbs and accessory olfactory bulbs) were isolated. Tissue samples were kept at -80 °C until proteomic processing.

2.3. Immunohistochemistry

For analysing c-Fos-like immunoreactivity, experimental subjects (10 wild-type and 10 nNOS-deficient mice) were separated

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