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RISC activity in hippocampus is essential for contextual memory

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ABSTRACT

RNA-Induced Silencing Complex (RISC) mediates post-transcriptional control of gene expression and contains Argonaute 2 (AGO2) protein as a central effector of cleavage or inhibition of mRNA translation. In the brain, the RISC pathway is involved in neuronal functions, such as synaptic development and local protein synthesis, which are potentially critical for memory. In this study, we examined the role of RISC in memory formation in rodents, by silencing AGO2 expression in dorsal hippocampus of C57BL/6 mice and submitting animals to hippocampus-related tasks. One week after surgery, AGO2 downregulation impaired both short-term and long-term contextual fear memories. Conversely, no long-lasting effects were observed three weeks after surgery, when AGO2 levels were re-established. These results show that altered RISC activity severely affects learning and memory processes in rodents.

MicroRNAs (miRNAs) are small non-coding RNA molecules which mediate post-transcriptional control of gene expression, through specific base pairing with the 3′ untranslated region (3′ UTR) of target mRNAs [5]. Functional miRNAs are incorporated into the RNA-Induced Silencing Complex (RISC), a ribonucleoprotein complex containing Argonaute (AGO) proteins as central effectors of the translation inhibition [10]. RISC may induce either mRNA cleavage or inhibition of mRNA translation depending on perfect or partial complementarity between the miRNA and its target mRNA [4,5]. In the central nervous system, miRNAs have been shown to play important roles in both neuronal development and neuronal functions [4,8,12]. Noteworthy, miRNAs are also found in dendrites where they likely regulate mRNA translation [14,15], suggesting a role in the regulation of local protein synthesis that is potentially critical for synaptic plasticity and memory [2,18–20]. Indeed, the involvement of RISC in memory formation has been demonstrated in *Drosophila* [3,6]. However, a direct evidence of its role in memory processes in mammals is still lacking.

The present study aims to investigate the involvement of RISC in memory formation in rodents, by downregulating AGO2 protein in dorsal hippocampus, a structure known to be crucial for contextual memory [1], and submitting animals to hippocampus-related tasks. Among the four members of mammalian subfamily of AGO proteins (AGO1–AGO4), AGO2 has been found to be expressed at the highest level in the brain [17], and it is the only Argonaute potentially affecting both translation and degradation of mRNA [10]. Noteworthy, AGO2 activity is essential for the RISC function to be effective [13].

To induce AGO2 downregulation, a pool of five different plasmids (0.5 μg/μl) expressing siRNAs (shAGO2 TRC 9630–9634, Sigma), specifically designed to target only AGO2 and not the other members of the AGO family of proteins, were complexed with polyethyleneimine (in vivo-jetPEI, Polyplus Transfection; Supplemental Fig. 1 online) and bilaterally injected (0.2 μl/site; rate 0.1 μl/min) into the dorsal hippocampus of C57BL/6 mice (shAGO2 group). A total of eight bilateral injections were performed. Coordinates in reference to bregma were [−] 2.2 anteroposterior (AP), [±] 1.5 mediolateral (ML), [−] 1.5 and [−] 2 dorsoventral (DV), [±] 2.5 ML, [−] 1.8 and [−] 2.7 DV. As a control vector, a scrambled siRNA was used (CV group; Supplemental Methods online). Previous evidence indicated that polyethyleneimine is able to mediate an efficient and selective gene delivery in neuronal cells of different brain regions, including cerebral cortex, hippocampus and hypothalamus [9,11,21]. Consistently with previous reports [11,21], we found that in time-course experiments the maximum

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expression of the reporter gene GFP is achieved one week after the injection (Supplemental Fig. 2 online). Thus, plasmid transfection, that induces a temporary expression of siRNAs, was used to evaluate possible long-lasting effects exerted by transient AGO2 downregulation on memory. After surgery, animals were allowed one week of recovery, then, two groups of shAGO2 ($n=8$) and CV ($n=9$) mice were submitted to an open field (60 cm diameter) to evaluate possible effects of AGO2 downregulation on both basal behaviors and anxiety levels. No significant differences between shAGO2 and CV mice were observed in locomotion ($F_{(1,15)}=0.044$; $p=0.83$), no locomotion ($F_{(1,15)}=0.07$; $p=0.79$), rearing ($F_{(1,15)}=0.23$; $p=0.64$), and freezing ($F_{(1,15)}=0.044$; $p=0.97$) as well as in the time mice spent close to the walls ($F_{(1,15)}=1.27$; $p=0.28$) and in the middle of the arena ($F_{(1,15)}=1.38$; $p=0.26$) (Supplemental Results and Supplemental Fig. 3 online). Memory was evaluated by submitting different groups of mice (shAGO2 $n=19$ and CV $n=17$) to the contextual fear conditioning task (Fig. 1A and Supplemental Methods online). This took place in a square conditioning chamber A (26 cm × 22 cm × 18 cm) with metal grid floor. Training lasted 180 s and consisted of a 120 s acclimatization period followed by a 30 s tone (conditioned stimulus, CS), overlapped in the last 2 s by a footshock (unconditioned stimulus, US; 0.5 mA). After CS–US administration, an additional 30 s period was allowed in order to evaluate footshock reaction. Test was carried out 30 min after training by re-exposing animals to the conditioning chamber A for 300 s, to investigate short-term contextual memory. Freezing behavior, defined as immobility except for respiration movements, was considered as a memory retention index. During training (Training A), shAGO2 and CV mice showed no significant differences in the percentage of freezing ($F_{(1,34)}=0.05$; $p=0.81$), and reacted alike to the US ($F_{(1,34)}=70.63$; $p<0.0001$). Conversely, shAGO2 mice showed a significant reduction of freezing behavior when tested for short-term memory (Test A, $F_{(1,34)}=13.46$; $p<0.001$), compared to CV mice (Fig. 1B, left). Short-term memory deficit was confirmed by submitting different groups of shAGO2 and CV mice to an inhibitory avoidance task. Animals were trained by administration of two consecutive footshocks (0.4 mA, 2 s, 5 s ISI) immediately after they moved from the light to the dark compartment of the apparatus, and tested 30 min post-training. Step-through latency in the test session, considered as a memory retention index, was significantly reduced in shAGO2 mice compared to CV ($F_{(1,30)}=5.34$; $p=0.02$) (Supplemental Methods, Supplemental Results and Supplemental Fig. 4 online).

In order to evaluate long-lasting effects due to transient AGO2 downregulation, two weeks after test in chamber A, shAGO2 ($n=9$) and CV ($n=8$) mice were re-trained in a modified conditioning chamber (chamber B; Supplemental Methods online) with the same procedure above described (see Fig. 1A). No differences between groups were observed in the freezing levels recorded during the training (Training B, $F_{(1,15)}=0.04$; $p=0.84$). Thirty minutes after re-training, short-term memory was tested by submitting animals to the same context B. No significant differences in the freezing levels were observed between the two groups of mice ($F_{(1,15)}=0.01$; $p=0.93$; Fig. 1B, right). The absence of long-lasting effects was further confirmed by submitting different groups of shAGO2 ($n=8$) and CV ($n=8$) mice to the training and test in conditioning chamber A three weeks after surgery (Fig. 1C). As shown in Fig. 1D, freezing level of shAGO2 mice was comparable to that of CV mice during both training ($F_{(1,14)}=0.2$; $p=0.66$) and short-term memory test ($F_{(1,14)}=0.68$; $p=0.79$).

As concerns long-term memory, different groups of shAGO2 ($n=8$) and CV ($n=8$) mice were submitted to long-term memory retention test in the fear conditioning task one week after surgery (Fig. 1E). As expected, when tested 24 h after training shAGO2 mice showed a severe long-term memory deficit ($F_{(1,14)}=9.45$; $p<0.01$) in comparison with CV mice (Fig. 1F).

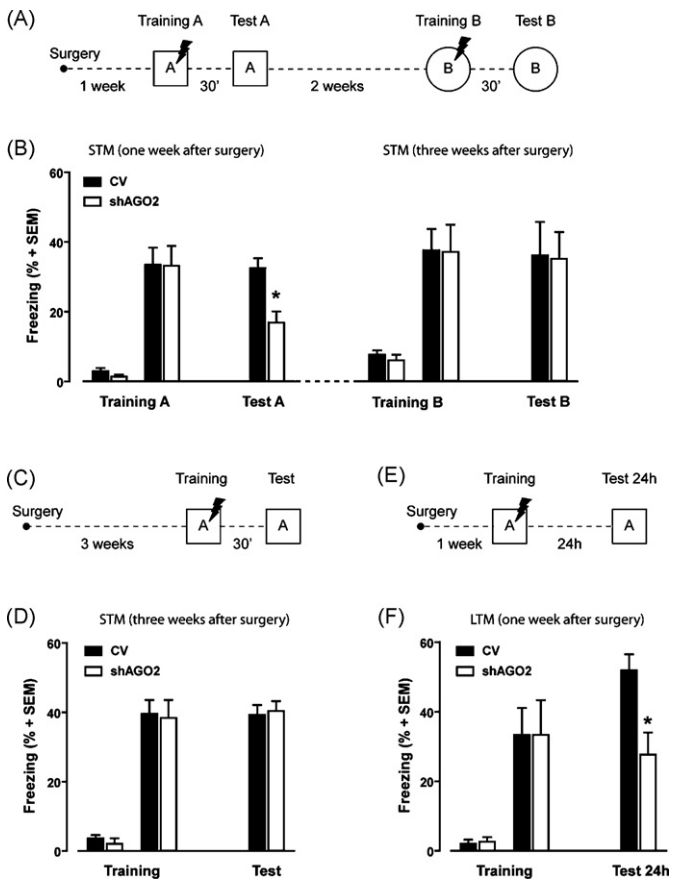

Fig. 1. Short-term and long-term memories are impaired in shAGO2 mice. (A) Experimental time line (short-term memory evaluation one week and three weeks after surgery). One week after surgery CV and shAGO2 mice were trained in the conditioning cage A (Training A). Short-term contextual memory was evaluated 30 min after training (Test A). Two weeks after Test A, CV ($n=8$) and shAGO2 ($n=9$) mice underwent a new training in the conditioning cage B (Training B). Short-term memory was again evaluated 30 min after training (Test B). (B, left) Percentage of time spent in freezing by CV ($n=17$; black bars) and shAGO2 ($n=19$; white bars) mice during training (Training A) and short-term memory test (Test A) one week after surgery. Concerning the training, two-way ANOVA showed no significant difference between groups (CV vs. shAGO2, $F_{(1,34)}=0.05$; $p>0.05$), a significant effect of US administration (pre-US vs. post-US, $F_{(1,34)}=70.64$; $p<0.0001$) and no significant interaction effect ($F_{(1,34)}=0.03$; $p>0.05$). Concerning the test, one-way ANOVA showed a significant difference between groups ($F_{(1,34)}=13.46$; $p<0.001$). (B, right) Percentage of time spent in freezing by CV ($n=8$) and shAGO2 ($n=9$) mice during re-training and short-term memory test in cage B two weeks after Test A. No significant differences between groups were observed in both Training B ($F_{(1,15)}=0.04$; $p>0.05$) and Test B ($F_{(1,15)}=0.007$; $p>0.05$). (C) Experimental time line (short-term memory evaluation three weeks after surgery in the contextual fear conditioning task). (D) Percentage of time spent in freezing by CV ($n=8$) and shAGO2 ($n=8$) mice during training and short-term memory test. Concerning the training, two-way ANOVA showed no significant difference between groups (CV vs. shAGO2, $F_{(1,14)}=0.2$; $p>0.05$), a significant effect of US administration (pre-US vs. post-US, $F_{(1,14)}=97.03$; $p<0.0001$) and no significant interaction effect ($F_{(1,14)}=0.003$; $p>0.05$). Concerning the short-term memory test, one-way ANOVA showed no significant difference between groups ($F_{(1,14)}=0.07$; $p>0.05$). (E) Experimental time line (long-term memory evaluation one week after surgery in the contextual fear conditioning task). (F) Percentage of time spent in freezing by CV ($n=8$) and shAGO2 ($n=8$) mice during training and long-term memory test. Concerning the training, two-way ANOVA showed no significant difference between groups (CV vs. shAGO2, $F_{(1,14)}=0.02$; $p>0.05$), a significant effect of US administration (pre-US vs. post-US, $F_{(1,14)}=25.713$; $p<0.0005$) and no significant interaction effect ($F_{(1,14)}=0.002$; $p>0.05$). Concerning the long-term memory test, one-way ANOVA showed a significant difference between groups ($F_{(1,14)}=9.45$; $p<0.01$). *$p<0.05$.

Immediately after short-term memory tests (carried out one week and three weeks after injection) shAGO2 and CV mice were sacrificed and the efficiency of AGO2 knockdown was assessed by both western blot analysis and real-time PCR. The immunoblot analysis, performed on proteins extracted from homogenates of