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 Figs. S1 to S7
 References
 Movies S1 and S2

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Role of Layer 6 of V2 Visual Cortex in Object-Recognition Memory

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Cellular responses in the V2 secondary visual cortex to simple as well as complex visual stimuli have been well studied. However, the role of area V2 in visual memory remains unexplored. We found that layer 6 neurons of V2 are crucial for the processing of object-recognition memory (ORM). Using the protein regulator of G protein signaling-14 (RGS-14) as a tool, we found that the expression of this protein into layer 6 neurons of rat-brain area V2 promoted the conversion of a normal short-term ORM that normally lasts for 45 minutes into long-term memory detectable even after many months. Furthermore, elimination of the same-layer neurons by means of injection of a selective cytotoxin resulted in the complete loss of normal as well as protein-mediated enhanced ORM.

The current dominant view of visual memory is the multiple-domain approach, under which different components of recognition memory such as perception, storage, famil-

ilarity, and recollection are subserved by different modules in the brain (1–5). Recognition memory, one of the most studied examples of declarative memory, is generally considered to consist of two

components, recollection and familiarity, and depends on the medial temporal lobe (MTL), a structure composed of the hippocampus and adjacent perirhinal, entorhinal, and parahippocampal cortices (6, 7). In contrast, perceptual learning is a domain-of-perception module localized outside the MTL, which includes ventral visual-stream structures such as area V2. However, the multiple-domain approach has been contradicted (7–10). It is argued that the entire ventral visual-to-hippocampal stream is important for visual memory (9). This

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Fig. 1. Animal performance on ORM task. **(A)** When an object was shown for 3 min, normal rats could retain the object information in memory for 30 and 45 min but not for 60 min. **(B)** Injection of lentivirus-RGS-14 into layer 6 of area V2 boosted the animal ORM capacity such that the animal was able to retain the same object information for more than 24 weeks. **(C)** The ability of RGS-14 animals to convert 45-min memory into long-term memory remained even after 14 months when a novel visual stimulus was presented. **(D)** Normal animals could retain information about two different objects (obj) but were unable to remember when four objects were shown. Animals with RGS-14 were able to keep in memory the information about six different objects shown. In all figures, the exploration time is derived from one old object and one new object except in (D), in which values are derived from multiple old objects, as indicated in the figure, and 1 new object. Delay is the interval between exploration trial and memory test trial (30). Values are presented as mean ± SEM obtained from number (n) of animals indicated beneath the bars. Asterisk indicates a significantly longer exploration time for a new object (P < 0.05).

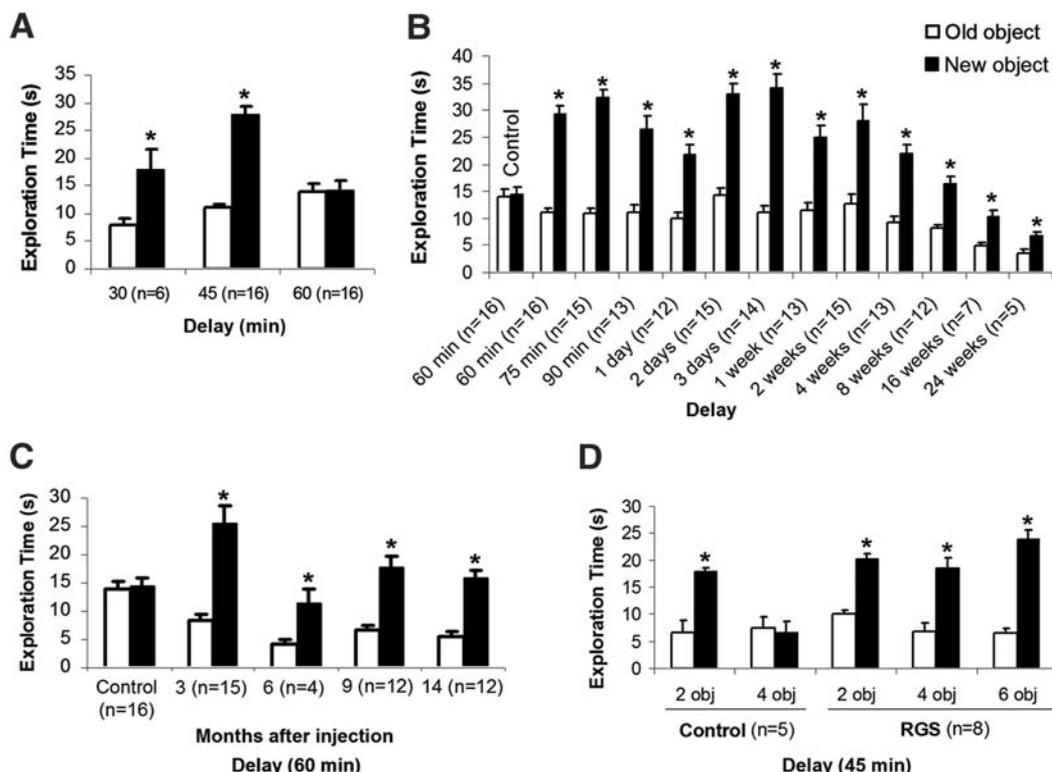
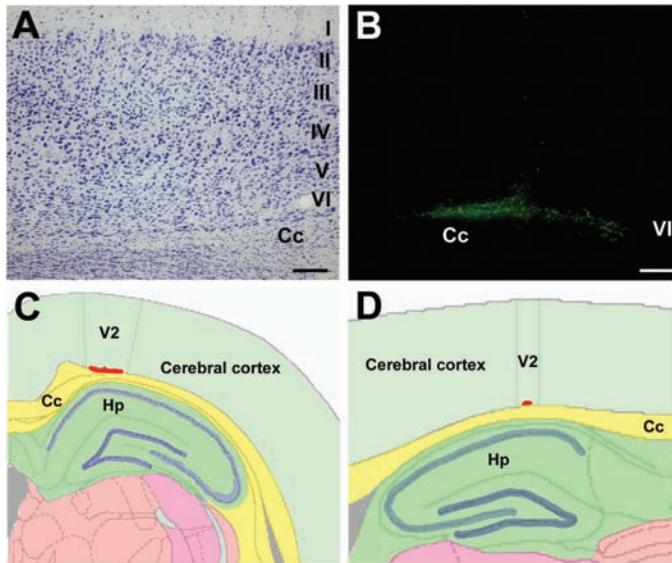


Fig. 2. Localization of RGS-14 protein in lentivirus–RGS-14–injected animals. **(A)** Cresyl violet staining of area V2 in the adjacent section indicates the corresponding layer 6. **(B)** A coronal brain section shows the localization of RGS-14 proteins as green fluorescence in layer 6 of the V2 visual cortex. **(C to D)** Drawings show localization of RGS-14 protein (red) at the injection site obtained from the analysis of coronal (*n* = 5 rat brains) **(C)** and sagittal (*n* = 7 rat brains) **(D)** serial sections. Both sections represent the maximum spread area observed. Area V2 is delimited by thin lines within the cerebral cortex in both **(C)** and **(D)**. Distribution of cortical layers from layer 1 (I) to layer 6 (VI) is shown in **(B)**. Cc, corpus callosum; Hp, hippocampus; scale bars, 125 μ m.



theory, unlike the dominant one, predicts that object-recognition memory (ORM) alterations could result from the manipulation in V2, an area that is highly interconnected within the ventral stream of visual cortices. In the monkey brain, this area receives strong feedforward connections from the primary visual cortex (V1) and sends strong projections to other secondary visual cortices (V3, V4, and V5) (11, 12). Most of the neurons of this area are tuned to simple visual characteristics such as orientation, spatial frequency, size, color, and shape (13–15). V2 cells also respond to various complex shape characteristics, such as the orientation of illusory contours (15) and whether the stimulus is part of the figure or the ground (16). Anatomical studies implicate layer 3 of area V2 in visual-information processing. In contrast to layer 3, layer 6 of the visual cortex is composed of many types of neurons, and their response to visual stimuli is more complex. But the importance of layer 6 in visual-information processing remains an enigma.

We used a multidomain protein known as regulator of G protein signaling–14 (RGS-14) to dissect out the role of area V2 in visual memory. This protein contains a conserved RGS domain, which binds active Gi/o α -guanosine triphosphate (α -GTP) to confer GTPase activating protein activity (17, 18), a GoLoco/GPR motif that binds to inactive Gi α -GDP and Gi α 1/3 (19–21), a tandem Rap1/2-binding domain (RBD) (22), and other regions with unknown functions (23, 24). RGS-14 protein is associated with microtubules and is an important factor in mitosis (25). Although the expression of RGS-14 protein was observed in monkey and rat brain (26), very little is known about its role in brain functions.

We used animal performance on ORM tests to evaluate visual memory. Rats were exposed to two identical objects for 3 min in an open field, and then they were analyzed for the length of time that object information was retained in their memory. During the ORM test session, one of the two identical objects was replaced with a new object, and the exploration time for both objects was recorded. After the memory test with delay periods of 30, 45, and 60 min, normal animals were able to retain object information for 30 and 45 min but not for 60 min (Fig. 1A). We called this limited visual memory of normal animals short-term ORM or normal ORM, and surpassing the normal ORM time limit was considered to be an increase in memory. Normal animals injected with lentivirus of RGS-14 into layer 6 of area V2 of rat brain 3 weeks before the test were subjected to the evaluation of ORM status. The overexpression of RGS-14 protein into layer 6 produced a large increase in the normal ORM (Fig. 1B). In contrast to normal animals, in which ORM lasted for 45 min, a visual stimulus of the same length of time to RGS animals led to the formation of long-term ORM, lasting for many months (Fig. 1B). Injection of lentivirus of RGS-12 (a protein that belongs to the same family as

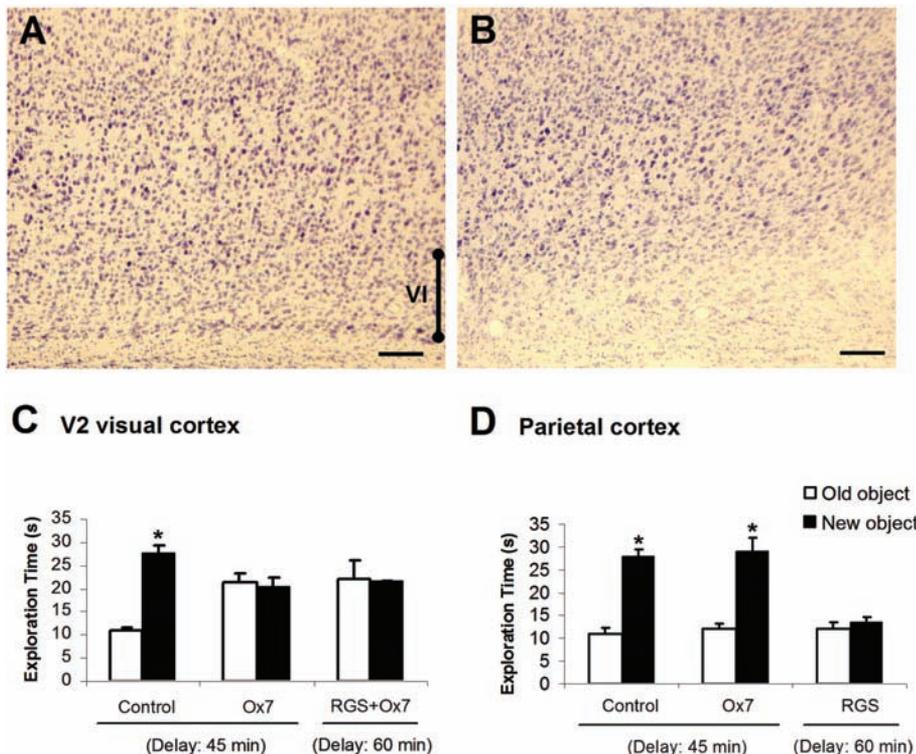


Fig. 3. Ox7-SAP injection in layer 6 and determination of ORM. **(A)** A normal view of layer 6 (VI) of the V2 visual cortex stained with cresyl violet. **(B)** Injection of Ox7-SAP (Ox7) into layer 6 of area V2 resulted in a major loss of neurons in this layer. **(C)** The behavioral performance of animals injected with Ox7-SAP **(B)** on the ORM task showed a complete loss of normal visual memory after 45 min. Similarly, when RGS-14 animals with confirmed enhanced ORM, as shown in Fig. 1, were treated with Ox7-SAP, the RGS-14–mediated effect on ORM was also abolished. **(D)** Injection of Ox7-SAP into the same layer of parietal cortex, an area just adjacent to V2, produced no effect on normal memory. Furthermore, in contrast to area V2, RGS-14–mediated effect on ORM was also absent in this area. Values are presented as mean \pm SEM obtained from 5 to 8 animals in each case. Asterisk shows significantly higher exploration time for a new object (*P* < 0.05).

RGS-14), saline solution, or vehicle lentivirus into the same area showed no such change in the ORM level. The performance of rats was similar to that of normal noninjected animals (fig. S1). The average exploration time during the initial 3-min exploration session of two identical objects was not significantly different between the vehicle-control (16.09 ± 0.81 s; $n = 16$ rats) and animals injected with RGS-14 (18.62 ± 1.47 s; $n = 15$ rats) (fig. S2). This finding excludes other factors than RGS-14 protein being involved into the memory boost. The injection of lentivirus of RGS-14 into the 2/3 layer of the V2 visual cortex, an area dorsal to the target site, and into CA1 and the dentate gyrus of hippocampus, areas ventral to the target site, did not produce an effect similar to that seen with the injection into layer 6 of the V2 visual cortex (fig. S3). Overexpression of RGS-14 protein in this layer not only boosted ORM but also maintained this characteristic for more than 14 months toward novel visual stimuli (Fig. 1C). In addition, the capacity to retain information on multiple objects was also higher in RGS animals. Normal animals could retain the information about two different objects but were unable for four objects. However, in contrast to normal animals, RGS animals could retain information about six objects (Fig. 1D).

After the behavioral experiments, brains of animals injected with lentivirus-RGS-14 were processed for immunocytochemistry to localize the area and/or site of protein expression in order to uncover the anatomical correlate of the memory enhancement observed in these animals. Brain sections were incubated with specific antibodies to RGS-14 (26) coupled with green fluorescence to visualize the protein. The expression of RGS-14 protein in area V2 was mainly localized in layer 6 (Fig. 2B). The analysis of injection site in sequential coronal ($n = 5$ rat brains) (Fig. 2C) as well as sagittal ($n = 7$ rat brains) (Fig. 2D) brain sections further confirmed the RGS-14 protein localization in layer 6 of area V2. Fig. 2, C and D, provides a 360° view of the RGS-14 protein localization area observed in the brain.

Next, we tested the importance of layer 6 neurons of area V2 in the formation of ORM by means of selective elimination of the neurons of this layer. Would the elimination of neurons in this layer abolish the both normal (short-term) and long-term memory mediated by the RGS-14 protein? We injected Ox7-SAP, a saporin-based immunotoxin that selectively eliminates neurons (27, 28), into layer 6 of area V2 and evaluated the visual memory of animals. Injection of the immunotoxin resulted in the loss of almost all the neurons of this area while leaving other cells and structure intact (Fig. 3B). However, no damage to the hippocampus was observed (fig. S4). Behavioral performance of the Ox7-SAP-injected animals on an ORM task showed a complete loss of normal ORM [delay of 45 min (Fig. 3C)], suggesting a major role of layer 6

neurons in visual memory. In addition to the facilitation in long-term ORM formation, layer 6 neurons are crucial for normal ORM. Furthermore, injection of Ox7-SAP in RGS animals abolished the protein-mediated enhancement in ORM [delay of 60 min (Fig. 3C)]. This result further confirmed the association of layer 6 neurons in the formation of long-term ORM. In contrast to area V2, injection of Ox7-SAP in layer 6 of parietal cortex, an area adjacent to area V2, did not produce any effect on normal ORM [delay of 45 min (Fig. 3D)]. Similarly, overexpression of RGS-14 protein in other areas, such as layers 2/3 of area V2, dentate gyrus and CA1 of the hippocampus (fig. S3), and layer 6 of the parietal cortex (Fig. 3D), also had no effect on long-term ORM (delay of 60 min).

The loss of both normal and RGS-mediated enhanced ORM after elimination of area V2 layer 6 neurons led us to question whether this layer of neurons serves as an ORM storage site. In RGS animals, after encoding information on novel objects, layer 6 neurons of area V2 were eliminated by Ox7-SAP treatment, and stored ORM was traced. Despite the absence of layer 6 neurons, these animals were able to recall information on objects shown before the Ox7-SAP treatment (fig. S5). However, their normal as well as RGS-mediated enhanced ORM were lost for novel visual stimuli (fig. S6). Thus, our results show that layer 6 of area V2 is implicated in ORM formation but not in its storage.

After passing through area V2, visual information continues ventrally through other visual areas to the MTL, a domain where ORM is thought to be processed (6, 7). Our findings of the role of layer 6 neurons in the formation of both normal (short-term) and long-term ORM provide a new dimension to our understanding and emphasize the importance of V2, an area localized outside of MTL. It is proposed that layer 6 neurons of area V2 modulate the processing of visual information flow by either direct or indirect intrinsic connections within this area from layer 6 to other layers. In accordance with our results, a magnetic resonance imaging study designed to map the functional activation of the visual cortices in response to visual stimulus (29) showed the activation of area V2 during both visual perception and recall. In conclusion, our results show that layer 6 of area V2, an area which previously was thought to be involved in perception and perceptual learning, not only plays a critical role in the formation of short- and long-term visual memory but also contradicts the multiple domain theory and supports the view that the entire stream of ventral visual-to-hippocampus, and not the MTL alone, is important for visual memory processing. Additionally, the role of RGS-14 protein in the enhancement of visual memory makes this protein an important pharmaceutical target for the treatment of ORM

defects as well as for boosting the memory capacity.

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