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Mg^{2+} Block of *Drosophila* NMDA Receptors Is Required for Long-Term Memory Formation and CREB-Dependent Gene Expression

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SUMMARY

NMDA receptor (NMDAR) channels allow Ca^{2+} influx only during correlated activation of both pre- and postsynaptic cells; a Mg^{2+} block mechanism suppresses NMDAR activity when the postsynaptic cell is inactive. Although the importance of NMDARs in associative learning and long-term memory (LTM) formation has been demonstrated, the role of Mg^{2+} block in these processes remains unclear. Using transgenic flies expressing NMDARs defective for Mg^{2+} block, we found that Mg^{2+} block mutants are defective for LTM formation but not associative learning. We demonstrate that LTM-dependent increases in expression of synaptic genes, including *homer*, *staufen*, and *activin*, are abolished in flies expressing Mg^{2+} block defective NMDARs. Furthermore, we show that genetic and pharmacological reduction of Mg^{2+} block significantly increases expression of a CREB repressor isoform. Our results suggest that Mg^{2+} block of NMDARs functions to suppress basal expression of a CREB repressor, thus permitting CREB-dependent gene expression upon LTM induction.

INTRODUCTION

Ca^{2+} enters a cell through NMDAR channels only when presynaptic glutamate release and depolarization of the postsynaptic membrane occur simultaneously (correlated activity). Conversely, NMDAR-mediated Ca^{2+} influx is suppressed at voltages near the resting membrane potential (uncorrelated activity), due to Mg^{2+} block, a mechanism in which the pore of NMDARs is blocked by external Mg^{2+} ions (Mayer et al., 1984; Nowak et al., 1984). Since Mg^{2+} block allows cells to discriminate between correlated synaptic inputs and uncorrelated activity, NMDARs have been proposed to function as "Hebbian coincidence detectors." However, the behavioral significance and molecular effects of Mg^{2+}-block-dependent suppression of Ca^{2+} influx during uncorrelated activity remains unknown (Single et al., 2000).

Functional NMDARs are heteromeric assemblies of an essential NR1 subunit and various NR2 subunits. Studies of NMDAR channels have demonstrated that Mg^{2+} block is dependent on an asparagine (N) residue at a "Mg^{2+} block site" located in a putative channel-forming transmembrane segment (TM2, see Figure 2A) of each subunit (Burnashev et al., 1992; Mori et al., 1992; Single et al., 2000). *Drosophila* have a single NR2 homolog, dNR2, which contains a glutamine at the Mg^{2+} block site (Q721), and a single NR1 homolog, dNR1, which contains an N at this site (N631). A previous study has shown that the N631 residue in dNR1 is sufficient for Mg^{2+} block in flies (Xia et al., 2005).

In *Drosophila*, early labile memory formed after Pavlovian olfactory associative learning is consolidated into two longer lasting memory phases: long-term memory (LTM), which requires new gene expression and protein synthesis, and anesthesia-resistant memory (ARM), which can be formed in the presence of transcriptional and translational inhibitors (Tully et al., 1994). In both vertebrates and invertebrates, a transcription factor, CREB, plays a critical role in gene expression required for LTM formation (Bourtchuladze et al., 1994; Yin et al., 1994). While previous studies have shown that hypomorphic mutations in *Drosophila* NMDARs (dNMDARs) disrupt both associative learning (LRN) and LTM formation without affecting ARM (Wu et al., 2007; Xia et al., 2005), it is still not clear how Mg^{2+} block is involved in these processes.

To understand the functional significance of Mg^{2+} block in dNMDARs, we generated transgenic flies expressing *dNR1* mutated at the Mg^{2+} block site, *dNR1(N631Q)*, in neurons. Strikingly, we found that these Mg^{2+} block mutant flies are defective for LTM formation but not LRN. We show that Mg^{2+} block functions to suppress basal expression of a repressor isoform of *Drosophila* CREB during uncorrelated activity. This allows increased CREB-dependent gene expression to occur during correlated activity, leading to formation of LTM.

RESULTS

Reduced Mg^{2+} Block in Transgenic Flies Expressing *dNR1(N631Q)*

Immunohistochemical studies using antibodies to dNR1 demonstrate that dNMDARs are expressed throughout the

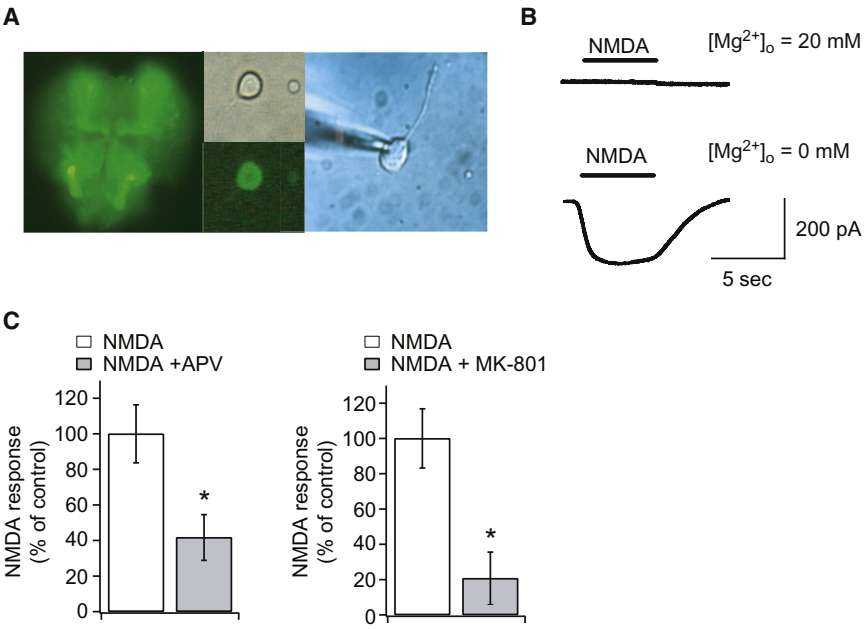

Figure 1. Physiological and Pharmacological Properties of Endogenous dNMDARs in the Fly Brain

(A) The pupal neuronal primary culture system. (Left) GFP image of neurons in an intact *elav/GFP* pupal brain. (Middle) A phase contrast image of a neuron dissociated from the pupal brain (upper) and its GFP signal (lower). (Right) Whole-cell clamping of a GFP-positive neuron. See also Figure S1 for dNR1 distribution.

(B) Inward currents induced by 100 μM NMDA in GFP-positive cells are abolished in the presence of 20 mM Mg^{2+}. GFP-positive cells responded to NMDA at 0 mM Mg^{2+}.

(C) Both NMDAR antagonists APV (10 μM) and MK-801 (10 μM) significantly decrease NMDA-activated currents in GFP-positive neurons. *$p < 0.05$ by t test. $n = 6$ for all data. Error bars in all figures in this paper indicate SEM.

Drosophila brain (Figure S1 available online) (Xia et al., 2005; Zachepilo et al., 2008; Zannat et al., 2006). Therefore, we used an *elav-GAL4/UAS-GFP* (*elav/GFP*) transgenic line (Brand and Perrimon, 1993), which expresses GFP in neurons, to characterize endogenous dNMDARs in pupal primary cultured neurons (Figure 1A). Using whole-cell patch clamp, we determined that more than 85% of GFP-positive cells showed NMDA-induced inward currents at a −80mV membrane potential in the absence of external Mg^{2+} (119 out of 136 cells, Figure 1B). These responses were blocked by physiological concentrations of 20 mM Mg^{2+} (Stewart et al., 1994). In addition, mammalian NMDAR antagonists, APV and MK801, significantly suppressed NMDA-activated currents (Figure 1C). These results demonstrate that endogenous dNMDARs are widely expressed in neurons of the fly brain and have similar physiological and pharmacological properties to mammalian NMDARs.

We overexpressed either wild-type *dNR1*(*wt*) or Mg^{2+}-block-site-mutated *dNR1*(*N631Q*) transgenes (Figure 2A) in neurons using an *elav-GAL4* driver: *elav-GAL4/UAS-dNR1*(*wt*), [*elav/dNR1*(*wt*)], and *elav-GAL4/UAS-dNR1*(*N631Q*), [*elav/dNR1*(*N631Q*)]. Overexpression of dNR1(wt) and dNR1(N631Q) proteins was confirmed by western blots (Figure S2). As seen in Figure 2B, all dNMDAR-mediated currents in neurons from *elav/dNR1*(*wt*) pupae showed significant Mg^{2+} block in the presence of Mg^{2+}, a result similar to what was seen in neurons from wild-type pupae. In contrast, Mg^{2+} block of dNMDAR-mediated currents was abolished in neurons from *elav/dNR1*(*N631Q*) pupae. I-V curves of *elav/dNR1*(*N631Q*) pupae in the presence or absence of Mg^{2+} were identical at membrane potentials of −80 mV or above, indicating that overexpression of *dNR1*(*N631Q*) dominantly suppresses Mg^{2+} block.

Notably, the *dNR1*(*N631Q*) mutation does not alter the dose-dependent responsiveness to NMDA in the absence of Mg^{2+} (Figure S3A). Furthermore, in contrast to *elav/dNR1*(*wt*) cells, NMDA-induced currents remain constant at different Mg^{2+} concentrations in *elav/dNR1*(*N631Q*) cells (Figure S3B), suggesting that the N631Q mutation alters Mg^{2+} sensitivity without altering channel pharmacology.

LTM Is Disrupted in *elav/dNR1*(*N631Q*) Flies

Hypomorphic mutations in *dNR1* ($dNR1^{EP3511}$ and $dNR1^{EP331}$) disrupt learning (LRN), memory measured immediately after aversive olfactory conditioning, and short-term memory (STM), assayed 1 hr after training (Figures 3A and 3B) (Xia et al., 2005). In contrast, both LRN and STM are normal in *elav/dNR1*(*N631Q*) flies, as well as in transgenic control *elav/dNR1*(*wt*) flies (Figures 3A and 3B).

To investigate the role of Mg^{2+} block in associative learning in more detail, we measured LRN after short-duration training, a modified short-program training protocol for which learning is plotted as a function of training duration (Cheng et al., 2001). As seen in Figure 4A, as training duration increases, LRN scores increase up to a maximum plateau. While this increase is inhibited in hypomorphic $dNR1^{EP3511}$ and $dNR1^{EP331}$ mutants, it is slightly enhanced in *elav/dNR1*(*N631Q*) flies. Strikingly, the LRN defects in hypomorphic *dNR1* mutants is rescued by expressing *dNR1*(*N631Q*) in neurons (Figure 4B), suggesting Mg^{2+} block may not be required for learning.

Mg^{2+} block has been proposed to restrict dNMDAR activation to cells receiving coincident stimulation. Thus, lack of Mg^{2+} block may activate dNMDARs in more neurons than is normal during olfactory conditioning, creating a situation in which the conditioned response may not be restricted to the conditioned odor. To test this possibility, we performed olfactory conditioning by pairing a single CS+ odor with electric shocks and then test measured escape responses to the CS+ odor as well as unrelated odors. As seen in Figure S4, when *elav/dNR1*(*N631Q*) flies are conditioned to OCT, avoidance of OCT increases compared to nonconditioned controls, while avoidance of MCH and benzaldehyde (BA) does not, suggesting