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Control of aversion by glycine-gated GluN1/GluN3A NMDA receptors in the adult Medial Habenula

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Abstract

The unconventional N-methyl-D-aspartate (NMDA) receptor subunits GluN3A/B can generate excitatory conductances purely activated by glycine, when associated with the other glycine-binding subunit GluN1. However, functional GluN1/GluN3 receptors have not been identified in native adult tissues. We discovered that GluN1/GluN3A receptors are operational in neurons of the mouse adult medial habenula (MHb), an epithalamic area controlling aversive physiological states.

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AUTHOR CONTRIBUTIONS

M.A.D. designed and developed the project. Y.O. and M.A.D. performed the electrophysiology with K.P., S.A.G., T.B. and E.S. K.P., C.V.R. and C.A. performed confocal immunohistochemistry, F.M. the EM under supervision of L.A. E.D. performed the behavioral tests under the supervision of B.L.K., with M.A.D. and K.P. T.G. performed the HEK experiments under supervision of P.P. S.D. contributed equipment. C.A. provided transgenic mice. M.A.D. wrote the manuscript, edited by all authors.

DATA AND MATERIALS AVAILABILITY

Data values and statistics are included as supplementary material.

COMPETING INTERESTS

Authors declare no competing interests.

In the absence of glycinergic neuronal specializations in the MHb, glial cells tuned neuronal activity *via* GluN1/GluN3A receptors. Reducing GluN1/GluN3A receptor levels in the MHb prevented place aversion conditioning. Our study extends the physiological and behavioral implications of glycine by demonstrating its control of negatively-valued emotional associations *via* excitatory glycinergic NMDA receptors.

ONE SENTENCE SUMMARY

Physiology of glycine NMDA receptors

Glycine is a major inhibitory neurotransmitter of the central nervous system, acting via anion-permeable receptors. It is also a well-characterized co-agonist of excitatory N-methyl-D-aspartate receptors (NMDARs) via GluN1 subunits (1).

In addition, glycine binds the unconventional NMDAR subunits GluN3A/B, which generate atypical glutamate-activated triheteromeric NMDARs with GluN1 and GluN2 subunits (2), and glycine-gated diheteromeric excitatory complexes with GluN1 (3). GluN3B is significantly expressed only in caudal areas (4). GluN3A expression is broader, but assumed to be limited to early development (2). Mainly examined in recombinant systems (4, 5), native GluN1/GluN3ARs have been identified in the juvenile hippocampus (8), but never in adult neurons.

We found strong immunohistochemical expression of GluN3A subunits in the ventral subdivision of the medial habenula (MHb) of adult mice (Fig.1A; (9)), an area mediating aversive behaviors (10–14).

We investigated GluN3A-immunostained sections with electron microscopy to identify its ultrastructural localization. Frequently in juxtaposition with glial structures, all the 3,3-diaminobenzidine (DAB)-labelled profiles were identified as postsynaptic dendrites and somata (n=92; Fig.1B). Supported by further confocal microscopy results (Fig.S1), these data demonstrated that GluN3A subunits were abundantly expressed in adult MHb neurons.

We examined whether GluN3A subunits formed operational GluN1/GluN2/GluN3ARs. These receptors show reduced rectification compared to GluN1/GluN2 NMDARs (1). We thus examined the current-voltage (I-V) curves of synaptic (17), and puff-evoked NMDAR currents in MHb neurons from wild type (WT) and GluN3AKO (15) mice, in which excitatory transmission to the MHb is not modified (Fig.S2). No significant differences were found (Fig.1Ca&Cb), suggesting absence of functional receptors.

To investigate whether glycine-activated GluN1/GluN3ARs were operational, glycine (1–10mM) was pressure-ejected while recording spontaneous firing activity (17) in the loose cell-attached configuration (LCA). In all tested WT neurons, glycine puffs (300–500ms) potently increased firing in the presence of D-2-amino-5-phosphonovalerate (D-APV) and strychnine (Fig.1D). This excitatory effect was not due to conventional NMDARs, because it was present in GluN2AKO mice (Fig.1D), where GluN1/GluN2 NMDARs are nearly undetectable (17). The effect on firing activity was absent in GluN3AKO mice (Fig.1D), supporting the presence of GluN1/GluN3ARs in the MHb.

Glycine puffs induced rapidly rising and inactivating inward currents in voltage clamp recordings of both WT and GluN2AKO *ex-vivo* MHb neurons performed in the presence of D-APV and strychnine. The currents could not be elicited in GluN3AKO mice (Fig.1E). Similarly to recombinant systems (5, 6), the glycine-evoked currents showed slight outward rectification (Fig.1G).

We investigated the pharmacology of the glycine-induced currents. In recombinant systems occupation of the higher affinity GluN3A site activates the receptor, whereas glycine binding to GluN1 entrains rapid desensitization (5, 6). We recently discovered that the GluN1 antagonist (1S)-1-[[(7-Bromo-1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxalinyl)methyl]amino]ethyl]phosphonic acid (CGP78608) enhanced receptor responses by reducing densitization (8). We found that CGP78608 (1μM) potentiated and prolonged the responses in WT but not GluN3AKO mice (Fig.2A). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), 5,7-Dichlorokinurenic acid (5,7-DCKA) and D-serine inhibit the currents produced by GluN1/GluN3Rs (3, 5, 6, 8). These drugs potently inhibited the responses to glycine also in MHb neurons (Fig.2B). Direct applications of D-serine increased neuronal excitability (Fig.S4) and elicited currents (Fig. 2C) to smaller extents than glycine (3). With the exception of zinc, which had no effects ((7); Fig.S5), the pharmacological profile of the native currents thus mirrored that of recombinant GluN1/GluN3Rs.

We injected the MHb with a short hairpin ribonucleic acid (shRNA)-expressing adeno-associated virus targeting the GluN3A subunit (AAV5-shRNA-GluN3A; (18)). The virus efficiently reduced both the messenger RNA levels of GluN3A in the MHb (Fig.2D), and the amplitude of the glycine-induced currents compared to scrambled RNA-expressing mice (Fig.2D).

The permeability of GluN1/GluN3ARs to calcium (Ca^{2+}) remains uncertain (3, 19). We examined this question by first analyzing in human embryonic kidney (HEK) cells a GluN1/GluN3AR variant with potentiated glycine responses (GluN1-F484A/GluN3A; (4, 7)). Increasing the extracellular Ca^{2+} concentration led to a shift of the reversal potential of the responses to glycine (Fig.2Ea), which demonstrated significant Ca^{2+} permeability, although smaller than for WT GluN1/GluN2A receptors (Fig.S6).

Using two photon microscopy in the presence of synaptic and Ca^{2+} channel blockers, we could detect glycine-evoked Ca^{2+} transients originating from GluN1/GluN3ARs in *ex-vivo* MHb cells (Fig.2Eb). We concluded that also native GluN1/GluN3A receptors were permeable to Ca^{2+}.

We then searched for physiological sources of glycine. We previously found that the MHb was completely devoid of glycinergic neuronal specializations (Fig.S1; (20)). We thus tested whether glial cells could control extracellular glycine levels in mice expressing the excitatory Designer Receptor Exclusively Activated by Designer Drugs (DREADD) hM3Dq under the control of the promoter of the glial fibrillary protein (GFAP). In these mice hM3DqR activation triggers Ca^{2+} elevations specifically in glial cells (21). We confirmed that hM3DqR expression was exclusively glial (Fig.3A&S7).

To determine whether GluN1/GluN3ARs mediated glia-neuron interactions in the MHb, we recorded *ex-vivo* spontaneous firing activity in GFAP-hM3Dq mice (Fig.3). Application of the hM3DqR agonist clozapine-N-oxide (CNO; 10μM), produced rapid firing rate increases.

Pre-incubation with 5,7-DCKA and CNQX strongly reduced the effect of CNO, suggesting a substantial contribution from GluN1/GluN3ARs (Fig.3). Interestingly, CGP78608 application significantly augmented basal firing rates (Fig.S3), indicating that ambient glycine may suffice to bind GluN3A. The potentiation of the firing rate triggered by CNO was instead reduced by CGP78608 pre-incubations.

Furthermore, the effect of CNO was significantly smaller in GFAP-hM3D mice injected with the AAV5-shRNA-GluN3A-GFP than with AAV5-scrRNA-GluN3A-GFP virus (Fig.3). In contrast to GlyT2 (Fig.S1), we found expression of the membrane glycine transporter GlyT1 in MHb glia (Fig.S8), which can contribute to glial glycine accumulation (22). In the presence of the GlyT1-specific blocker N-[(3R)-3-([1,1'-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine (ALX5407), CNO applications increased firing rates to greater values than in control (Fig.3C&D), likely because of greater build-up of extracellular glycine levels. Together these experiments suggested that glial cells potentiated neuronal activity *via* activation of GluN1/GluN3ARs.

We finally examined whether GluN1/GluN3ARs in the adult MHb were behaviorally relevant, in mice injected with either the AAV5-shRNA-GluN3A-GFP, or the AAV5-scrRNA-GluN3A-GFP virus (Fig.S9). First, no differences were detected in locomotor and exploratory activity in an open field arena (Fig.4A). In contrast the test mice spent significantly less time in the open arms of an elevated place maze, suggesting that reduced GluN1/GluN3AR levels in the MHb were mildly anxiogenic (Fig.4A&B).

GluN1/GluN3ARs in the MHb did not modulate learning and memory capabilities because test and control mice scored similarly in a novel object recognition test (Fig.4C).

The MHb can contribute to the development of negatively-valued emotional states. We thus examined the behavioral outcomes of a fear conditioning protocol (Fig.4D). We found no difference between AAV5-shRNA- and AAV5-scrRNA-injected mice in the freezing time elicited either by the cued, or the contextual stimulus (Fig.4D).

Finally, we examined lithium-induced conditioned place aversion (CPA), because this paradigm relies on intact MHb function (23, 24). In contrast to AAV5-scrRNA animals, lithium-treated AAV5-shRNA mice did not develop aversion for the conditioned compartment (Fig.4E). Overall, these results indicate that impaired GluN3A signaling in the MHb can alter the capability of associating negatively-valued external conditions with internal aversive states.

In addition to its ubiquitous role as inhibitory neurotransmitter, glycine can thus exert excitatory actions via unconventional GluN1/GluN3A NMDARs. Our study unveils a functional role of this aspect of glycine physiology by demonstrating that full expression of GluN1/GluN3ARs in the MHb is mandatory for modifying the internal emotional landscape in response to specific environmental challenges.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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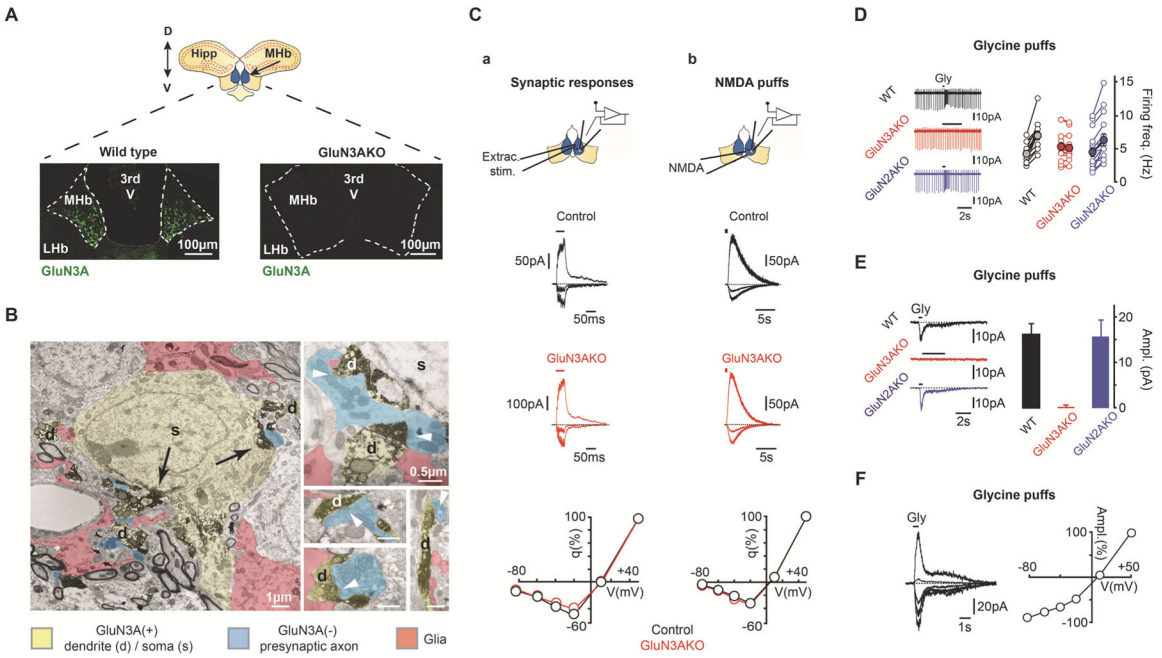

Fig.1. Functional GluN1/GluN3ARs in the adult MHb.

Both confocal (**A**) and EM (**B**) demonstrated that GluN3A subunits are expressed in the ventral MHb. DAB EM staining (**B**, black deposits) was detected in dendrites (yellow compartments, **d**), close to glial extensions (red), rarely in somas (black arrows, **s**), but never in axons (blue, arrowheads highlighting presynaptic specializations). (**C**)GluN1/GluN2/GluN3ARs are not functional in the MHb. No difference was found in the rectification of NMDAR currents in WT (black) and GluN3AKO (red) mice, elicited by extracellular stimulation (**a**) or pressure-ejected NMDA (**b**). (**D**)Glycine puffs increased firing rates in MHb cells from control and GluN2AKO mice, but not GluN3AKO animals. (**E**, **F**)Similarly to heterologous GluN1/GluN3R currents, glycine induced outwardly rectifying, rapidly rising and inactivating currents in control and GluN2AKO, but not GluN3AKO neurons. Data are illustrated as averages±s.e.m.

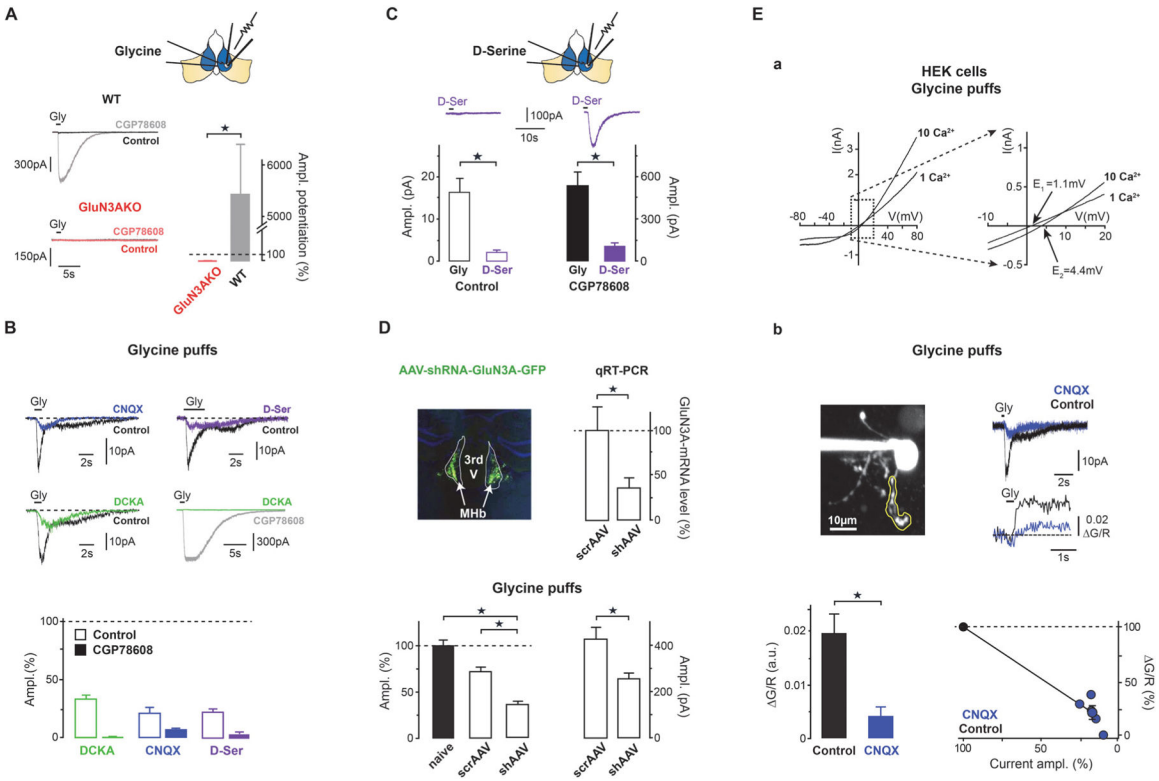

Fig.2. Properties of GluN1/GluN3Rs in the MHb.

(**A**)GluN1 subunit block with CGP78608 potentiated glycine-elicited currents. (**B**)Bath applications of CNQX (blue), 5,7-DCKA (green) and D-serine (purple) antagonized control and potentiated GluN1/GluN3AR currents. (**C**) Pressure-ejected D-serine had only partial agonist effects on GluN1/GluN3ARs. (**D**)Viral expression in the MHb of a GluN3A-targeting shRNA led to reduction of GluN3A mRNA levels, and of glycine-induced currents with respect to scrambled RNA-expressing mice. Percentage values and absolute amplitudes are illustrated in the lower graphs. (**E**)GluN1/GluN3ARs are Ca^{2+} permeable. Changing extracellular Ca^{2+} concentrations led to a significant reversal potential shift (**a**, highlighted on the right) in heterologous systems expressing GluN3A and point-mutated, glycine-insensitive GluN1 subunits. In the presence of synaptic receptor antagonists and Ca^{2+} channel blockers, two-photon imaging revealed glycine-elicited Ca^{2+} transients in *ex-vivo* MHb neurons (**b**). Data are illustrated as averages±s.e.m.

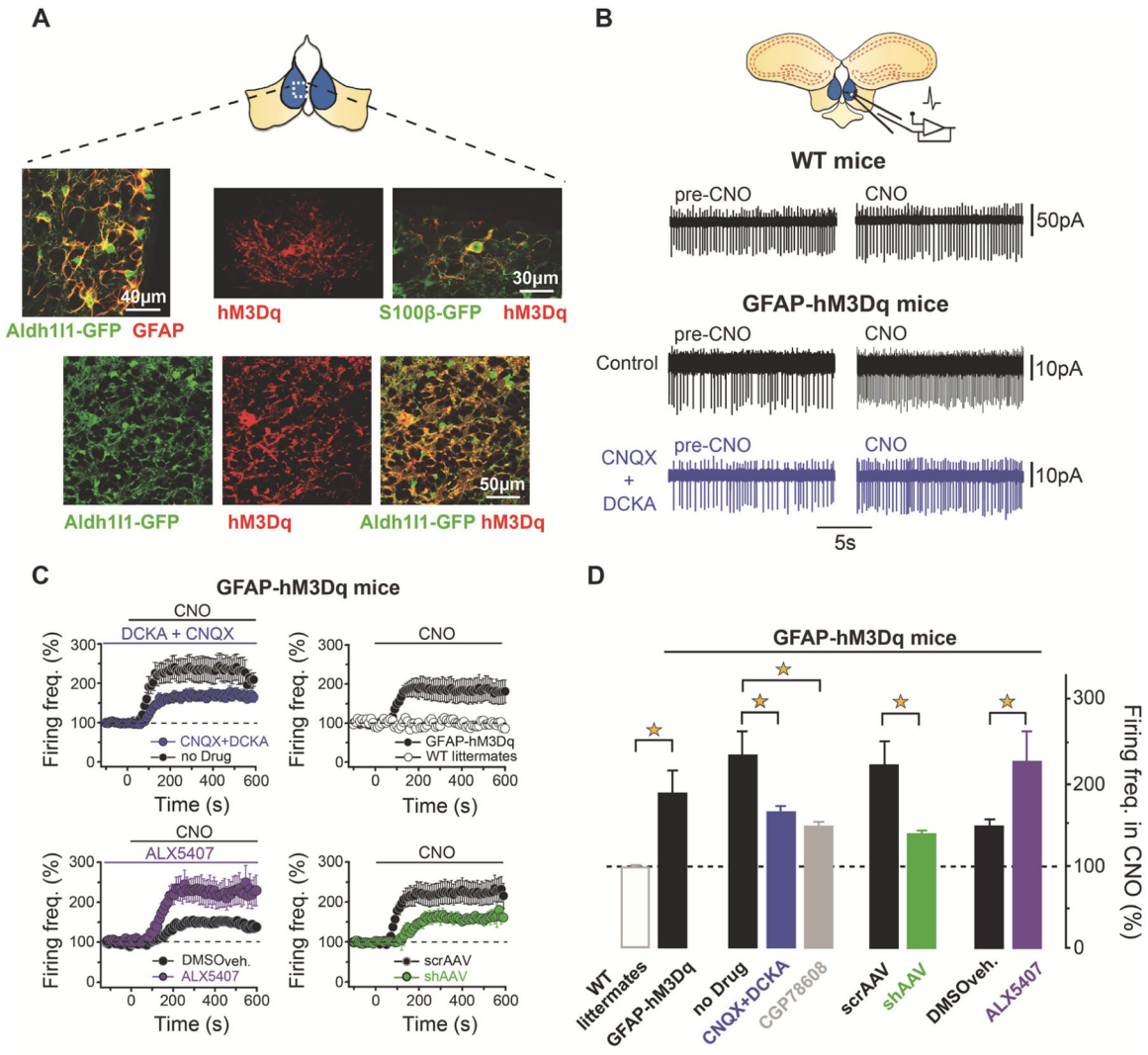

Fig.3. Glial activation in GFAP-hM3Dq mice increases neuronal excitability via GluN1/GluN3ARs.

(**A**)The specific expression of hM3Dq receptors in MHb glia was demonstrated by the colocalization of GFAP and GFP in aldehyde dehydrogenase-1 family member L1-green fluorescent protein (Aldh1l1-GFP) mice, and of hM3DqRs and GFP in GFAP-hM3Dq::Aldh1l1-GFP and GFAP-hM3Dq::S100β-GFP mice. (**B**)CNO applications increased spontaneous firing rates of MHb neurons. Representative traces preceding and following CNO are shown for specified experiments. The average time courses (**C**) and quantifications (**D**) of the CNO effects are depicted for all the experiments. The smaller increase of the firing rate in CGP78608 than in control likely derives from reduced affinity for glycine in the drug presence ((8)). Data are illustrated as averages±s.e.m.

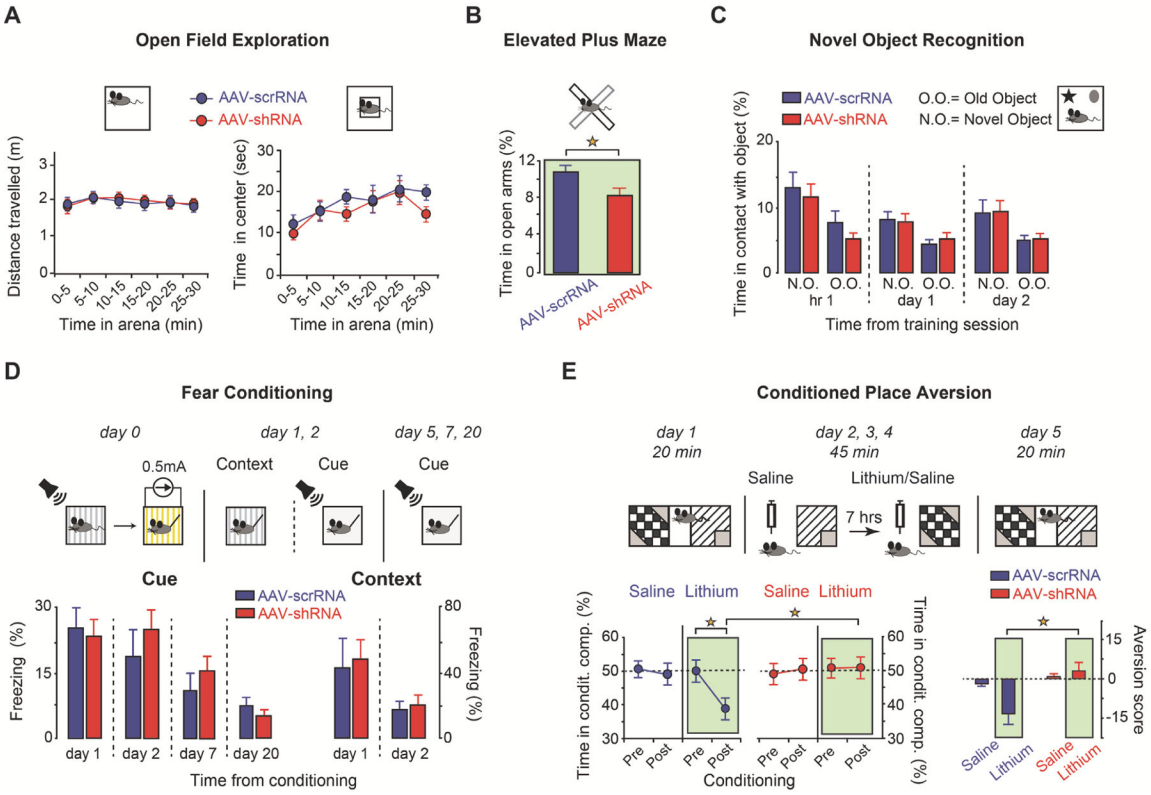

Fig.4. MHb GluN1/GluN3ARs control place aversion conditioning.

(**A**)No difference was detected between AAV-shRNA-GluN3A- and AAV-scrRNA-GluN3A-injected mice in the distance traveled in an open field and in the time spent in its center. (**B**)Knocking-down MHb GluN3A expression was mildly anxiogenic as indicated by the shorter time spent by GluN3A-deficient mice in the open arms of an elevated plus maze. Object exploration times and cue- and context-induced freezing were similar in novel object recognition (**C**) and fear conditioning tests (**D**), respectively. Following lithium conditioning, AAV-shRNA-GluN3A-injected mice developed no aversion for the malaise-associated compartment in contrast to scrRNA-expressing animals (**E**). Data are illustrated as averages ±s.e.m.