Kainate receptor auxiliary subunit NETO2 is required for normal fear expression and extinction

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Abstract

NETO1 and NETO2 are auxiliary subunits of kainate receptors (KARs). They interact with native KAR subunits to modulate multiple aspects of receptor function. Variation in KAR genes has been associated with psychiatric disorders in humans, and in mice, knockouts of the *Grik1* gene have increased, while *Grik2* and *Grik4* knockouts have reduced anxiety-like behavior. To determine whether the NETO proteins regulate anxiety and fear through modulation of KARs, we undertook a comprehensive behavioral analysis of adult $Neto1^{-/-}$ and $Neto2^{-/-}$ mice. We observed no differences in anxiety-like behavior. However, in cued fear conditioning, $Neto2^{-/-}$, but not $Neto1^{-/-}$ mice, showed higher fear expression and delayed extinction compared to wild type mice. We established, by in situ hybridization, that *Neto2* was expressed in both excitatory and inhibitory neurons throughout the fear circuit including the medial prefrontal cortex, amygdala, and hippocampus. Finally, we demonstrated that the relative amount of synaptosomal KAR GLUK2/3 subunit was 20.8% lower in the ventral hippocampus and 36.5% lower in the medial prefrontal cortex in $Neto2^{-/-}$ compared to the $Neto2^{+/+}$ mice. The GLUK5 subunit abundance was reduced 23.8% in the ventral hippocampus and 16.9% in the amygdala. We conclude that *Neto2* regulates fear expression and extinction in mice, and that its absence increases conditionability, a phenotype related to posttraumatic stress disorder and propose that this phenotype is mediated by reduced KAR subunit abundance at synapses of fear-associated brain regions.

Introduction

NETO1 and NETO2 are CUB-domain containing proteins that interact with native kainate receptor (KAR) subunits. Defined as auxiliary subunits, they modulate functional properties of KARs, including

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desensitization kinetics and synaptic currents [1-4], and postsynaptic abundance [4-6]. NETO1 and NETO2 interact with cytoplasmic scaffolding proteins through their PDZ-ligand domain to form stable macromolecular complexes with KARs [1, 5]. In addition to KARs, NETO1 is an auxiliary subunit of N-methyl-D-aspartate receptor (NMDAR) [1] while NETO2 interacts with K-Cl co-transporter 2 (KCC2) [7].

Although NETO1 and NETO2 are highly homologous, their brain expression patterns differ considerably. NETO2 expression is highest in the cerebellum where it plays a crucial role in determining KAR subunit composition at the postsynaptic density [5]. The expression of NETO1 is highest in the hippocampus (Hpc) [3], where it regulates KAR-mediated excitatory post-synaptic currents [4, 8] and axonal targeting of KARs [9]. Recently Wyeth et al. [8] showed that both NETO1 and NETO2 tonically inhibit cholecystokinin (CCK)-expressing interneurons and that NETO1 regulates KAR-mediated excitation of interneurons in the developing Hpc. However, the roles of NETO1 or NETO2 in other brain regions have not been investigated thus far. Consequently, little is known about the contribution of the NETO proteins to complex behaviors. In accordance with the central role of the Hpc in the regulation of spatial memory, $Neto1^{-/-}$ mice have decreased spatial memory both in the Morris water maze (MWM) and displaced object (DOR) tasks [1], while the behavioral phenotypes of $Neto2$-/- mice have not been previously investigated.

KARs are members of the ionotropic glutamate receptor (iGluR) family, which mediates fast excitatory neurotransmission in the central nervous system. They are composed of five subunits, designated GLUK1-5 (encoded by $Grik1-5$ genes), which localize to distinct brain regions, cell types, and subcellular compartments [10-13]. In addition to their postsynaptic actions that are similar to the other iGluR family members, KARs modulate presynaptic neurotransmitter release at both excitatory [14-16] and inhibitory synapses [17, 18]. Variants in the $GRIK2$ gene have been associated with obsessive-

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compulsive disorder [19] and variants in *GRIK5* with bipolar disorder [20]. Moreover, significant decreases in the expression levels of *GRIK1* and *GRIK2* subunits have been reported in entorhinal and perirhinal cortices from bipolar disorder, major depression, and schizophrenia patients [21]. In mice, knockouts of the *Grik1* gene have increased anxiety-like behavior [22] while *Grik2* and *Grik4* knockouts have reduced anxiety-like behavior [23, 24].

Considering the importance of NETO proteins in the modulation of KAR function, we hypothesized that they may regulate anxiety behavior and undertook a comprehensive behavioral screen of anxiety-like and fear-related behaviors in *Neto1*$^{+/+}$, *Neto1*$^{-/-}$, *Neto2*$^{+/+}$ and *Neto2*$^{-/-}$ mice. We demonstrated that NETO2 is required for normal fear expression and extinction in cued fear conditioning, phenotypes present in human anxiety disorder patients [25]. Moreover, in fear-related brain regions, we found both that *Neto2* was expressed in excitatory and inhibitory neurons, and that the abundance of KAR subunits GLUK2/3 and GLUK5 was reduced at synapses in *Neto2*$^{-/-}$ mice. Altogether these results establish that NETO2 is required for normal fear expression and extinction in mice, and this phenotype may be mediated by reduced synaptic KAR abundance in the fear circuit.

Materials and Methods

Animals

Neto1 and *Neto2* knockout (KO) mice were generated as described before [1, 4] and were 8-12 weeks old at the time of experiments. The tested wild-type (WT) and KO mice were littermates, and the experimenter was blind to their genotype during behavioral testing. Mice were maintained at the Laboratory Animal Centre (LAC) of the University of Helsinki in Finland or at the Toronto Centre of Phenogenomics (TCP) in Canada. Animal procedures were approved by the project authorization board of the Regional State Administration Agency for Southern Finland and carried out in accordance to

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directive 2010/63/EU of the European Parliament and of the Council and the Finnish Act on the

Protection of Animals Used for Science or Educational Purposes (497/2013), and by the Animal Care

and Use Committee of the TCP in accordance with the requirements of the Province of Ontario

Animals for Research Act 1971 and the Canadian Council on Animal Care. See Supplementary

Material and Methods for details.

Behavioral testing

Elevated plus maze (EPM) test. Mice were placed in the center zone of an elevated (40 cm) plus-shaped

Plexiglass maze composed of two open and two closed arms (length 30 cm, width 5 cm, closed arm

wall height 15 cm) and video-tracked for 5 min using EthoVision XT10 software (Noldus,

Wageningen, Netherland).

Open field (OF), Forced Swim (FST), and Elevated zero maze (EZM) tests were carried out as

previously [26]. FST was also used as a stressor before measurement of blood corticosterone levels,

thus 30 min after the test mice were anesthetized with 4% isoflurane and blood samples were collected

from the submandibular vein. In the EZM, the risk assessment zone was determined as 5 cm at the

border of closed and open areas.

Light/dark box (LD) test. The same chamber as used for the OF was divided in light and dark

compartments (27 cm x 13 cm x 20 cm) with a hole in between. Mice were placed in the dark

compartment and allowed to explore the compartments for 5 min.

Contextual fear conditioning was performed as previously described [27] using the TSE system.

Context A was a transparent chamber (23 cm x 23 cm x 35 cm) with a grid floor delivering foot shock

[unconditioned stimulus (US), 0.6 mA, 2s]. During acquisition 3 x US were delivered (ITI 60-90 s after

180 s habituation period). Context memory retrieval was assessed by exposure to the same context for 300 s and freezing was measured with threshold of 2 seconds.

Cued fear conditioning and extinction were performed using the same system as contextual fear conditioning. During acquisition in context A, a sound cue [conditioned stimulus (CS), 76 dB, pulsed 5Hz, 30s] was delivered 3 times [inter-trial-interval (ITI) 30 s] followed by a US (0.6 mA, 2s). Context memory retrieval was assessed the next day by exposure to the context A without CS for 240 s. Two hours later, cue memory retrieval was tested by presenting 2 CS x 60 s in the same chamber but with black wall and white floor, and a layer of wood chips (bedding material) placed under the removable floor to give a distinctive odor to the context (context B). On the third day, extinction of cued fear conditioning was carried out by presenting 20 x 30 s CS (ITI 2s) in context B. Percent time freezing during extinction was analyzed as an average of 4 CS presentations. Extinction retrieval was measured 24 h later by presenting 4 x 30 s CS (ITI 2 s) in context B. To assess group differences in the rate of extinction learning we calculated a percentage of extinguished mice using Kaplan Meier survival analysis to normalize for the higher baseline freezing observed between genotypes. Freezing at the beginning of extinction (CS 1-4) was taken as the 100% freezing value for each animal. Then percent time freezing during each CS presentation was considered as survival (above 50% freezing) or no survival (below 50% freezing).

Morris water maze (MWM) and *Spatial and Novel object recognition tasks* were conducted at the TCP with male mice, as previously [1]. See Supplementary Material and Methods.

Spontaneous alternation task was conducted as previously described [28] using a 7-trial task with 30 s delay interval (6 alternations) in a grey Plexiglas T-shaped maze. Alternation was graded by giving a value of 1 if an alternation happened between two consecutive trials or a value of 0 if the mouse did not

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alternate. The sum of these values was considered the total alternation score. Alternation (%) was calculated by dividing the alternation score by the maximum score value (alternation score / 6 x 100).

Stress-induced hyperthermia (SIH) was assessed by rectal temperature measurement using a lubricated rectal thermometer probe. Temperatures were taken at t1 = 0 min and t2 = 10 min between which mice were returned to the home cage. The first temperature measurement indicated baseline temperature and represented the stressful event. Stress-induced hyperthermia was calculated by t2 – t1.

Saccharin preference (SP) was assessed in home cages. On the first day, animals were habituated to the drinking tubes. During the three following days mice had free access to both water and 0.5% saccharin bottles, which were inversed every day to avoid side preference bias. Tube weight (g) was measured each testing day to calculate liquid consumption. Saccharin preference score (%) was calculated by saccharin consumption / total liquid consumption x 100.

Home cage activity, acoustic startle reflex (ASR) and *hot plate (HP) test* see Supplementary Material and Methods.

Corticosterone ELISA assay

Blood samples were left undisturbed for 15-30 min at room temperature to clot, and then centrifuged at 1,000-2,000 g for 10 min at 4°C. Resulting serum was collected immediately and stored at -80°C. ELISA assay was performed as instructed in the Corticosterone High Sensitivity EIA kit (Immunodiagnostic Systems, The Boldons, U.K).

In situ hybridization (ISH)

Mice were injected with a lethal dose (600 mg/kg) of pentobarbital (Mebunat Vet 60 mg/ml, Orion Pharma) and transcardially perfused with phosphate buffer (PBS), followed by 4% paraformaldehyde (PFA) in PBS, both at 37°C. Brains were post fixed 2-4 days in PFA at 4°C, embedded in paraffin

blocks and sectioned (10 µm) using a Leica RM2255 microtome (Leica Biosystems, Nussloch, Germany). Nonradioactive ISH using probes coupled with either digoxin (DIG) or fluorescein (F) was performed as previously described [29], see Table S1 and Supplementary Material and Methods for details. Brain sections were imaged using Axio Imager 2 (Carl Zeiss, Oberkochen, Germany), processed and exported with Zeiss Zen Lite Software, and background was adjusted with Photoshop software (Abode, Mountain View, CA, USA). Imaging was restricted to specific bregma coordinates: mPFC between 1.9 and 1.7, dHpc and Amg between -1.5 and -2.0, and vHpc between -2.9 and -3.3.

Brain lysates and synaptosomal fraction enrichment

Brain regions of interest (see Figure 6a) were dissected, snap frozen, and stored at -80°C. mPFC and Amg were dissected using a micropunch (needle gauge 16). For lysates, tissue was homogenized in RIPA buffer [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl pH=8.0, and protease cocktail (Sigma-Aldrich, Saint-Louis, MI, USA)] using Precellys24 homogenizer (BertinInstruments, Montigny-le-Bretonneux, France), incubated 1-2 h under constant agitation at 4°C and centrifuged for 20 min at 12,000 rpm. Synaptosomal fraction was obtained as previously described [30]. mPFC and Amg samples were pooled (n=2-5 animals/pool).

Immunoblot analysis

Proteins were size-separated by electrophoresis using 4-20% acrylamide precast gels and transferred to a nitrocellulose membranes (BioRad, Hercules, CA, USA). Membrane was incubated for 1 h at room temperature in blocking solution (TBST + 10% non-fat milk powder or 3-5% BSA, depending on the primary antibody) prior to overnight incubation with primary antibody at 4°C: rabbit anti-NETO2 (1:1,000; 10% milk, gift from Dr. Roderick R. McInnes), mouse anti-PSD-95 (1:1,000 in TBST + 3% BSA, #sc-32290, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-synaptophysin (1:300, 5%

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BSA, #S5768, Sigma-Aldricht, Saint-Louis, MO, USA), rabbit anti-GLUK2/3 (1:1,000, 10% milk, #04-921, Millipore, Billerca, MA, USA), rabbit anti-GLUK5 (1:1,000, 10% milk, #06-315, Millipore, Billerca, MA, USA) and mouse anti-b-actin (1:2,000, 10% milk, #A1978, Sigma-Aldricht, Saint-Louis, MO, USA). They were then washed and incubated with secondary antibody at room temperature: goat anti-rabbit or goat anti-mouse HRP depending on the primary antibody (1:5,000 in primary antibody saturation solution, #115-035-144 and #115-035-146, Jackson ImmunoResearch, West Grove, PA, USA). Signal was visualized using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA, USA) and membranes were read using SynGen gel doc system G:Box (Syngen, Frederike, MD, USA) or Biospectrum imaging system (Analytik Jena US LLC, Upland, CA, USA). Protein band intensity was quantified using ImageJ version 1.47v (National Institutes of Health). Band intensity was first normalized with the b-actin signal intensity from the corresponding lane. In Figure 6c the protein amount of the synaptosomal fraction was then normalized to the homogenate protein amount. The homogenate and synaptosomal fraction samples were always analyzed on the same blot. Ratio of *Neto2*^{-/-} / *Neto2*^{+/+} protein abundances in Figure 6e were calculated as previously [4, 5]. All samples (KO: Cb n=7, vHpc n=7, mPFC n=4, AMG n=6; WT: Cb n=7, vHpc n=7, mPFC n=5, Amg,n=6) from the same brain region were ran on the same gel. Each gel run was repeated two times, resulting in three blots per brain region. All blots were analyzed for GLUK2/3, GLUK5, and b-actin signal intensities. GLUK2/3 and GLUK5 signal intensities were then normalized to the b-actin signal intensity. Within each blot, we then calculated the mean normalized signal intensities of all KO and all WT samples, and calculated their ratio (KO/WT). Statistical significance was calculated using one sample t-test comparing ratios from the three independent blots of each brain region to a reference value of one (this reference value corresponds to equal protein abundance in KO and WT). For full uncropped blots see Figure S4.

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Statistical analysis

Statistical analyses were conducted using SPSS Statistics 24 (IBM, Armonk, NY, USA),

GraphPadPrism7 software (GraphPad Software, San Diego, CA, USA), or RStudio (RStudio Inc.,

Boston, MA, USA). Mean ± SEM was determined for each group. Data were analyzed using Student's

t-test, mixed ANOVA, ANCOVA, repeated measure ANOVA, or Wilcoxon matched-pairs signed rank

test, and corrected for multiple comparisons using the Benjamini-Hochberg (BH) method [31]. We

report adjusted p-values (p_{adj}) defining significance as $p_{adj} < 0.05$. For more details, see Table S2 and

Supplementary Materials and Methods.

Results

Innate anxiety-like behavior in $Neto1^{-/-}$ and $Neto2^{-/-}$ mice is comparable to $Neto1^{+/+}$ and

$Neto2^{+/+}$ littermate controls

To determine whether either NETO protein has a role in anxiety-like behavior, we tested male and

female $Neto1^{-/-}$, $Neto1^{+/+}$, $Neto2^{-/-}$, and $Neto2^{+/+}$ mice in four paradigms that measure approach-

avoidance conflict [elevated plus maze (EPM), elevated zero maze (EZM), light/dark box (LD), and

open field (OF) tests]. In the EPM, we found no difference in the time spent in the open or closed arms,

or distance traveled in the closed arms between $Neto1^{-/-}$ and $Neto1^{+/+}$ or $Neto2^{-/-}$ and $Neto2^{+/+}$ mice

(Figure 1a and b, and Figure S1a). However, there was a trend that $Neto2^{-/-}$ female mice spent less time

(19.5%, nominal p=0.012) and moved a shorter distance in the closed arms (23.4%, nominal p=0.020)

compared to the $Neto2^{+/+}$ mice, although these differences did not survive multiple testing correction.

In the EPM, mice have to cross the center zone of the maze to go from one arm to another, and the time

spent in the center can confound the analysis. Thus, as we observed these trends, we also tested $Neto2^{-/-}$

and $Neto2^{+/+}$ in the EZM, which does not have a center zone. $Neto2^{-/-}$ mice did not differ from the wild

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type mice in the time spent in the open areas (Figure 1c) or in the risk assessment zones (Figure S1b), but *Neto2*$^{-/-}$ females moved again a shorter distance in the closed areas (Figure S1c, $p_{adj}=0.015$). In the LD, we did not observe any differences between *Neto1*$^{-/-}$ and *Neto1*$^{+/+}$ or *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice in time spent in the light compartment, latency to enter the light compartment, or the distance traveled in the dark compartment (Figure 1d and e, and Figure S1d). In the OF, neither *Neto1*$^{-/-}$ nor *Neto2*$^{-/-}$ differed from the wild type mice in the time spend in the center zone (Figure 1f), but as in the EZM, we observed reduced activity in the periphery of the chamber in female *Neto2*$^{-/-}$ compared to *Neto2*$^{+/+}$ mice (Figure S1e, $p_{adj}=0.022$).

Since differences in locomotor activity can confound measurement of anxiety-like behavior, we next measured home cage activity of *Neto1*$^{-/-}$, *Neto1*$^{+/+}$, *Neto2*$^{-/-}$, and *Neto2*$^{+/+}$ mice. No differences were observed between the groups (Figure S1f). Thus, the reduced activity of *Neto2*$^{-/-}$ mice in the EZM and OF appears to be related to novel environment. To further investigate the basis of the decreased activity in the novel environment of *Neto2*$^{-/-}$ mice, we measured stress-related behavior [stress-induced hyperthermia (SIH)], stress-induced plasma corticosterone (CORT) levels, and depression-like behavior [saccharin preference and forced-swim test (FST)] in *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice. We identified no differences between the genotypes in any of these tests (Figure 1g-j). Overall, these results demonstrate that *Neto2* is not required for normal physiological responses to stress or depressive-like behavior.

Contextual fear conditioning in *Neto1*$^{-/-}$ and *Neto2*$^{-/-}$ mice

We next asked whether *Neto1* or *Neto2* influence conditioned fear-related behaviors, and measured contextual fear conditioning using a simple task that creates an association between an unconditioned stimulus (US; electric footstock) and the context (Figure 2a). During the acquisition phase we measured freezing levels (i.e. fear expression in mice) before (pre-US) and after each US presentation

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(post-US1, post-US2, and post-US3). We did not observe any statistically significant differences in the freezing levels between $Neto1^{-/-}$ and $Neto1^{+/+}$ or $Neto2^{-/-}$ and $Neto2^{+/+}$ mice during the acquisition phase (Figure 2b, c, d and e). However, we observed a trend for $Neto2^{-/-}$ male mice for increased response during fear learning (nominal p=0.013, p_{adj}=0.055), but this did not result in increased memory retention (Figure 2d). The following day, we tested context retrieval by placing the mice back in the same context. Only $Neto1^{-/-}$ females demonstrated higher fear expression compared to controls (Figure 2c, Cx ret p_{adj}=0.032). Altogether, these findings suggest that $Neto2$ does not influence context retrieval after contextual fear conditioning, but that absence of $Neto1$ in female mice leads to enhanced fear memory retrieval after contextual fear conditioning.

$Neto2$ is required for normal cued fear expression and extinction

We next assessed cued fear conditioning in $Neto1^{-/-}$, $Neto1^{+/+}$, $Neto2^{-/-}$, and $Neto2^{+/+}$ mice (Figure 3a). In this task a sound cue (conditioned stimulus, CS) is associated with a footstock (US) and thus becomes a predictor of the US. In the acquisition stage, we found that $Neto2^{-/-}$ mice showed higher freezing levels compared to $Neto2^{+/+}$ mice (Figure 3b and c, males p_{adj}=0.009 and females p_{adj}=0.018), whereas no differences were observed between $Neto1^{-/-}$ and $Neto1^{+/+}$ mice during acquisition (Figure 3d and e).

On the following day we tested context retrieval in the same context in which mice experienced the CS-US association (context A), and cue retrieval in a new context (context B). $Neto2^{-/-}$ mice showed higher freezing levels compared to $Neto2^{+/+}$ mice during the context retrieval (Figure 3b and c, males p_{adj}=0.023 and females p_{adj} =0.043), whereas $Neto1^{-/-}$ and $Neto1^{+/+}$ did not differ in their freezing levels (Figure 3d and e). In the cue retrieval, $Neto2^{-/-}$ mice again froze more than $Neto2^{+/+}$ mice (Figure 3b and c, males p_{adj}=0.019 and females p_{adj} =0.018), but there were no differences between $Neto1^{-/-}$ and $Neto1^{+/+}$ animals (Figure 3d and e). To determine how much the genotype effect on cue retrieval

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depended on acquisition of fear, we reran the analysis using the freezing level at the end of the

acquisition phase (post-CS3) as a covariate. Genotype effects on cue recall were removed, suggesting

that the genotype effects on cue recall day were related to level of fear learning acquired rather than

increased recall per se.

We also measured fear extinction by presenting the CS 20 times without the US. We recorded freezing

before (pre-CS) and during each CS (CS1-20) and analyzed the results in five blocks of four CS. *Neto1*$^{-/-}$ and *Neto1*$^{+/+}$ mice displayed no differences in freezing levels during extinction or in extinction

efficiency (Figure 3d and e). In contrast, female *Neto2*$^{-/-}$ mice showed significantly higher freezing

levels and male *Neto2*$^{-/-}$ mice showed a trend to increased freezing levels compared to *Neto2*$^{+/+}$ mice

(Figure 3b and c, males $p_{adj}=0.054$ and females $p_{adj}=0.023$). Male *Neto2*$^{-/-}$ mice failed to extinguish the

fear memory during the five blocks of extinction while females were successful to extinguish (Figure

3b and c, 1st *vs* 5th block in males *Neto2*$^{+/+}$ $p_{adj}=0.021$ and *Neto2*$^{-/-}$ $p_{adj}=0.44$, and females *Neto2*$^{+/+}$

$p_{adj}=0.021$ and *Neto2*$^{-/-}$ $p_{adj}=0.026$). To take into account the overall higher fear expression of *Neto2*$^{-/-}$

mice, we performed a survival analysis (Figure 3f, g, h and i) that allowed us to normalize for the

freezing levels. Briefly, for each mouse we defined extinction as a reduction of freezing to 50% of the

freezing level at the beginning of extinction [CS(1-4)]. In accordance with their similar freezing levels

throughout the extinction, *Neto1*$^{-/-}$ and *Neto1*$^{+/+}$ did not show difference in the efficiency of extinction

(Figure 3f and g). However, fear extinction of *Neto2*$^{-/-}$ mice was significantly less efficient compared to

the *Neto2*$^{+/+}$ mice (Figure 3h and i, males $p_{adj}=0.0017$ and females $p_{adj}=0.009$). Finally, on the

following day we tested extinction retrieval. During this test, only female *Neto2*$^{-/-}$ mice demonstrated

higher freezing levels compared to *Neto2*$^{+/+}$ mice (Figure 3b, c, d and e, $p_{adj}=0.046$).

To examine whether the overall increased freezing in *Neto2*$^{-/-}$ mice could result from higher baseline

startle levels, we measured acoustic startle reflex (ASR). *Neto2*$^{-/-}$ males showed lower ASR levels

compared to the *Neto2*$^{+/+}$ mice (Figure S2a, p_{adj}=0.023), suggesting that *Neto2*$^{-/-}$ mice are not generally more sensitive to startle. To rule out the possibility of higher freezing due to increased pain sensitivity, we performed the hot plate test, but found no differences between the genotypes, showing that *Neto2*$^{-/-}$ mice are not more sensitive to pain stimuli than their *Neto2*$^{+/+}$ littermates (Figure S2b). Altogether, these results demonstrate less efficient fear extinction in the absence of *Neto2*, despite the overall higher freezing levels that are not due to higher pain or startle sensitivity.

Brain regions that regulate cued fear conditioning in mice are well established and include mPFC, Hpc, and Amg [32]. Their individual contributions may be distinguished using behavioral tasks that depend on specific brain regions. To characterize the brain regions involved in the *Neto2*$^{-/-}$ fear phenotype we performed the Morris water maze (MWM), displaced and novel object recognition (DOR and NOR), and T-maze tasks, which measure Hpc-dependent spatial memory (MWM and DOR) [33, 34] , Hpc- , Amg- and entorhinal cortex-dependent novelty recognition (NOR) [35], and Hpc- and mPFC-dependent spontaneous alternation (T-maze) [36, 37]. In the MWM, no differences were observed between genotypes in the time spent swimming in any of the quadrants during the probe trial (Figure 3j), including the SE quadrant (p_{adj}=0.38) that contained the platform during the training phase. In accordance with this result, *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice both recognized the displaced objects (DOs) from the non-displaced objects (NDOs) (Figure 3k, DO *vs* NDO: *Neto2*$^{+/+}$ p_{adj}=0.009, *Neto2*$^{-/-}$ p_{adj}=0.040). However, in the NOR task *Neto2*$^{+/+}$ mice successfully differentiated between the familiar (FO) and novel object (NO) while *Neto2*$^{-/-}$ failed to do so (Figure 3k, FO *vs* DO *Neto2*$^{+/+}$ p_{adj}=0.009 vs *Neto2*$^{-/-}$ p_{adj}=0.43). Finally, we did not observe working memory deficits in the T-maze as both *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice showed alternation above chance level (Figure 3l). In conclusion, these experiments establish that in addition to the enhanced cued fear expression and extinction phenotype, *Neto2*$^{-/-}$ mice have a deficit in recognition of novel objects, a Hpc-, Amg- and entorhinal cortex-dependent behavior.

Thus, based on the behavioral phenotype of the *Neto2*⁻ᐟ⁻ mice, which is restricted to specific tasks, we next asked where and in which cell types *Neto2* is expressed in the fear-related brain regions (mPFC, Amg and Hpc).

Neto2 is expressed in both excitatory and inhibitory cells in fear-related brain regions

To better understand the mechanisms related to the behavioral phenotype of the *Neto2*⁻ᐟ⁻ mice, we investigated *Neto2* expression pattern that remains poorly characterized. Previous studies have shown that it is highly expressed in the cerebellum [3, 5], and that in the Hpc it co-localizes with major KAR subunits in interneurons [8]. To determine the expression pattern of *Neto2* in fear-related brain regions [i.e. mPFC, dorsal and ventral Hpc (dHpc and vHpc), and Amg, Figure 4a-d], we carried out *in situ* hybridization (ISH) on *Neto2*⁺ᐟ⁺ mice and used *Neto2*⁻ᐟ⁻ mice as controls due to the relatively low level of signal (Figure 4 and Figure S3). We first established that *Neto2* was expressed in each studied brain region (Figure 4e-p). To identify the specific types of neurons that express *Neto2* in these locations, we carried out double ISH using markers for excitatory (vesicular glutamate transporter 1, *Vglut1*) or inhibitory (glutamate decarboxylase 1, *Gad1*) neurons (Figure 5). In the mPFC, *Neto2* was expressed in the cingulate (cg1), prelimbic (PL), and infralimbic (IL) cortices (Figure 4e-g, Figure S3a). In the dHpc and vHpc, *Neto2* was expressed in the CA1, CA3 and dentate gyrus (DG) (Figure 4h-m, Figure S3b and c). Finally, in the Amg, *Neto2* was expressed in both lateral (LA) and basolateral nucleus (BLA) (Figure 4n and o, Figure S3d). We did not detect any *Neto2* expression in the central nucleus (CE, Figure S3d), known to be crucial for fear expression in rodents [38, 39]. In intercalated cells (ITCs) of the Amg, *Neto2* expression was present in few cells (Figure 4p, Figure S3d). In all four brain regions, *Neto2* was expressed in both excitatory and inhibitory cells (Figure 5). To determine how many *Neto2*-expressing cells were inhibitory or excitatory neurons, we counted the number of *Neto2*-positive cells that also express *Gad1* or *Vglut1* (Figure 5 d, h, l and p). A larger proportion of *Neto2*-expressing cells

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in the Cg1, IL, and CA1 were inhibitory than excitatory, while in the DG, vCA1, vCA3, vDG, LA, and BLA the *Neto2*-expressing cells were mostly excitatory. As expected, *Neto2* positive cells were *Vglut1*-negative in the ITCs (Figure 5p) that are only composed of inhibitory cells. They are involved in fear memory extinction together with IL sub-region of the mPFC [40, 41]. Altogether, these results establish that *Neto2* is widely expressed in fear-related brain regions, suggesting that other factors than its expression pattern determines the specificity related to its function.

Neto2 ablation reduces KAR subunit abundance at synapses in fear-related brain regions

To assess if the behavioral phenotype of *Neto2*$^{-/-}$ mice could be due to altered abundance of its binding partners in specific cellular compartment, we investigated KAR subunits in the synapses of *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice. NETO2 interacts with native KAR subunits *in vivo* and *Neto2*$^{-/-}$ mice have reduced amount of GLUK2 subunits at cerebellar post-synaptic density (PSD) [5]. To test whether *Neto2*$^{-/-}$ mice have altered KAR subunit abundance in synapses of fear-related brain regions, we obtained synaptosomal fraction (pre- and post-synaptic regions) from mPFC, vHpc, and Amg using differential ultracentrifugation. Furthermore, we also examined the cerebellum (Cb), where *Neto2* is highly expressed, as a technical control for synaptosomal fraction enrichment. We first measured protein abundance of NETO2, GLUK2/3, and GLUK5 in lysates of the four brain regions (Figure 6a and b). We selected GLUK2/3 and GLUK5 subunits since they are the major KAR subunits in the mouse brain [12] and specific antibodies for them are available. We validated that NETO2 was absent from *Neto2*$^{-/-}$ tissue, and we did not observe differences between the genotypes in KAR subunit levels (see representative bands in Figure 6b). Next, we examined the synaptic enrichment by comparing Cb homogenate (H) and synaptosomal fraction (SYN) protein levels in *Neto2*$^{+/+}$ tissue (Figure 6c). We demonstrated enrichment in the SYN of both pre- and post-synaptic markers [synaptophysin (SYP) p=0.012 and PSD-95 p=0.002, respectively] and our proteins of interest (NETO2 p=1.1E^{-6}, GLUK2/3

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p=0.0006, and GLUK5 p=6.5E^{-7}), but not the ubiquitous control protein b-actin (Figure 6c). In the vHpc *Neto2*$^{-/-}$ mice had lower levels of GLUK2/3 (20.8%, p=0.0053) and GLUK5 (23.8%, p=0.026) in the SYN fraction compared to the *Neto2*$^{+/+}$ mice (Figure 6d and e). In the mPFC of *Neto2*$^{-/-}$ the reduction was even larger for both GLUK2/3 (36.5%, p=0.038) and GLUK5 (39.5%), although due to variation between replicates not statistically significant (p=0.059). In the Amg, the difference of GLUK2/3 abundance was not significantly different (29%, p=0.075) but the GLUK5 amount was reduced by 16.9% (p=0.0014) (Figure 6d and e). We did not find differences between *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice in the Cb (Figure 6d and e). In conclusion, our results establish that *Neto2* is required for normal abundance of major KAR subunits in the synapses of fear-associated brain regions, but not in cerebellum, a region not included in the main fear network.

Discussion

We have demonstrated that *Neto2* is required for expression and extinction of cued fear memories. This finding was specific to *Neto2* and to cued fear conditioning, since *Neto1*$^{-/-}$ mice did not have this phenotype and *Neto2*$^{-/-}$ mice did not have deficits in contextual fear conditioning. Furthermore, we established that neither *Neto1* nor *Neto2* regulate anxiety-like behavior in tests that measure approach-avoidance conflict. However, *Neto2*$^{-/-}$ female mice had reduced activity during these tests, suggesting that NETO2 may influence adaptation to a novel environment in females. We found that the abundance of major synaptic KAR subunits was reduced in fear-related brain regions of *Neto2*$^{-/-}$ vs. *Neto2*$^{+/+}$ mice. Altogether, these results indicate that NETO2 is critical for fear-related behaviors in mice, and that its effect on fear expression and extinction may be mediated by modulation of KAR subunit abundance at synapses in fear-related brain regions.

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Although *Neto1* and *Neto2* are homologues, their influence on anxiety and fear-related behaviors differed profoundly. The *Neto1*^{-/-} mice did not differ from the *Neto1*^{+/+} mice in anxiety-like behaviors, and they did not have deficits in cued fear conditioning. However, in contextual fear conditioning *Neto1*^{-/-} females froze more to the context during retrieval compared to the *Neto1*^{+/+} mice, suggesting stronger contextual fear memory in the absence of NETO1. It has been previously shown that *Neto1*^{-/-} mice have a learning deficit in both MWM and DOR tasks but that they are able to distinguish between familiar and novel objects during the NOR task [1]. *Neto1* expression levels are highest in the Hpc [3], which concurs with the absence of *Neto1* influencing contextual fear conditioning, MWM, and DOR that are all Hpc-dependent tasks.

In the *Neto2*^{-/-} mice, we observed significantly increased fear expression and delayed fear extinction in cued fear conditioning compared to the *Neto2*^{+/+} mice. *Neto2*^{-/-} mice showed higher freezing levels in response to the CS even after the first sound-shock presentation. We determined that the higher freezing levels of *Neto2*^{-/-} vs. *Neto2*^{+/+} mice was not due to higher pain or sensory sensitivity related to the sound cue. The higher fear expression phenotype in *Neto2*^{-/-} mice was specific to cued fear conditioning as we did not observe increased fear expression in these mice during the acquisition or retrieval in the purely contextual fear conditioning. We also observed delayed fear extinction in *Neto2*^{-/-} mice compared to the *Neto2*^{+/+} mice even when normalizing for higher freezing levels at recall. Higher fear expression during acquisition of fear memory in mice is reminiscent of higher fear conditionability in humans. Conditionability refers to the situation in which individuals are more prone to acquire conditioned responses to a traumatic unconditioned stimulus. Both higher conditionability and delayed extinction of CS have been observed in post-traumatic stress disorder (PTSD) patients [42-44], indicating that *Neto2*^{-/-} mice have a PTSD-like phenotype, since they display both of these key PTSD traits.

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Anxiety-like behavior, as assessed by the EPM, EZM, LD, and OF that measure approach-avoidance conflict, was normal in the *Neto2*^{-/-} mice. However, we found that female *Neto2*^{-/-} mice had reduced activity in novel environments (i.e. EZM and OF) but not in a familiar environment (i.e. home cage) compared to the wild type mice. Since a novel environment represents a stressful situation, this novelty-induced activity phenotype could result from dysregulation of the stress axis and thus affect other stress-related behaviors or physiology. However, we did not observe any differences between *Neto2*^{-/-} and *Neto2*^{+/+} mice in other stress or depression-like behaviors, including stress-induced hyperthermia, plasma corticosterone levels, despair-behavior in the FST, or anhedonia as measured by saccharin preference. Even though there were no genotype effects, some of these stress-related phenotypes differed between males and females, although we did not formally test for sex differences due to multiple testing burden. To conclude, *Neto2* appears not to be required for innate anxiety, stress-response, or depression-like behavior, but may influence adaptation to novel environments in females.

To further dissect the role of NETO2 in behavioral phenotypes involving the brain regions that regulate fear memory, we further investigated *Neto2*^{-/-} mice in mPFC-, Hpc-, and Amg-dependent tasks. The Amg is central for emotional learning, and processes threatening sensory information [32, 45]. The Hpc is responsible for contextual encoding of fearful situations [46, 47], and the mPFC regulates fear expression and extinction through projections to the Amg and Hpc [48, 49]. *Neto2*^{-/-} mice had no deficits in the Hpc-dependent MWM and DOR spatial tasks [33, 34]. These results are consistent with the lack of context retrieval deficits during cued and contextual fear conditioning in *Neto2*^{-/-} mice. In the NOR task, *Neto2*^{-/-} mice failed to distinguish between familiar and novel objects, which suggest a deficit in the Hpc, Amg, and/or entorhinal cortex functions [35]. Finally, *Neto2*^{-/-} mice alternated above chance level in the T-maze, suggesting that *Neto2* ablation does not affect Hpc- and mPFC-dependent spontaneous alternation [36, 37]. The brain networks involved in spatial memory, novel object

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recognition, and spontaneous alternation tasks are potentially different from those involved in fear conditioning. Our results demonstrate that NETO2 may contribute to circuits underlying novel object memory, such as Hpc, Amg, and entorhinal cortex.

Although *Neto2* is widely expressed throughout the brain, with the highest level in the cerebellum [3, 5], little is known about its expression pattern in fear-related brain regions. We established that *Neto2* was expressed in both excitatory and inhibitory neurons in fear-related brain regions mPFC, dHpc, vHpc, and Amg. This broad expression pattern does not provide additional information to elucidate the mechanisms by which *Neto2* regulates fear-related behaviors. Rather, since NETO2 interacts with many scaffolding proteins at both excitatory and inhibitory synapses to form stable macromolecular complexes, it is likely that its specific functions are regulated by such interactions and their subcellular localization [50]. In accordance with this concept, we established that abundances of KAR subunits GLUK2/3 and GLUK5 were 20 to 40% lower at synapses of fear-related brain regions (vHpc, mPFC, and Amg) but not of Cb of *Neto2*$^{-/-}$ mice. This reduction was specific to synaptic compartments, since the total protein abundance of GLUK2/3 and GLUK5 were unaffected by *Neto2* ablation in all studied brain regions (Cb, vHpc, mPFC, and Amg), as previously reported in Hpc and Cb [4, 5]. The lower abundance may be due to a default in KAR delivery and/or stability at synapses in the absence of NETO2 [4, 5, 8]. In the *Neto1*$^{-/-}$ dHpc, major KAR subunit abundance is lower in the PSD, and both KAR- and NMDAR-mediated excitatory post-synaptic currents are reduced [4]. Similarly, the reduced GLUK2/3 and GLUK5 abundance we observed in the synapses of fear-related brain regions in *Neto2*$^{-/-}$ mice may cause the reduction of KAR-mediated synaptic transmission, leading to higher fear expression and delayed extinction.

In conclusion, we have demonstrated that the homologous genes *Neto1* and *Neto2* have distinct roles in the regulation of behavior. We established that *Neto2* is important for fear-related behaviors and that its

ablation leads to higher fear expression and extinction deficits, a PTSD-like phenotype. In contrast, *Neto1* regulates Hpc-dependent spatial learning memory [1], consistent with its high expression levels in the Hpc. Although we did not observe robust differences in anxiety-like behavior, the absence of *Neto2* influenced adaptation to novel environment in females, the startle reflex in males, and novel object recognition, which we only measured in males. In keeping with its modulation of KAR function, *Neto2* is required to maintain the normal abundance of major KAR subunits at synapses in fear-related brain regions, which may mediate the ability to emotionally process threat cues. Altogether, these findings provide new insight into the role of KAR auxiliary subunit NETO2 in complex behaviors such as fear expression and memory. Our observations suggest the possibility that both KAR and NETO2 function may be compromised in human disorders associated with fear expression and extinction impairment. Identification of the underlying mechanisms of these phenotypes may lead to better understanding of the biological drivers behind anxiety- and fear-related disorders, a requirement for the development of targeted therapies.

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Figure legends

Figure 1. No difference in anxiety-like behavior between *Neto1*$^{-/-}$ and *Neto1*$^{+/+}$ or *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice. Results from the elevated plus maze (**a, b**), the elevated zero maze (**c**), the light/dark box test (**d, e**), the open field test (**f**), the stress-induced hyperthermia (**g**), the stress-induced plasma corticosterone (CORT) levels (**h**), the saccharin preference (**i**), and the forced swim test (**j**). Each dot represents one animal, M=males and F=females. Mean +/- 1 standard error is shown. Genotype effect calculated using t-test. KO=knock out, WT=wild type.

Figure 2. Contextual fear conditioning in *Neto1*$^{-/-}$, *Neto1*$^{+/+}$, *Neto2*$^{-/-}$, and *Neto2*$^{+/+}$ mice. Schematic of the protocol (**a**). Freezing levels of *Neto1* male (**b**) and female (**c**), and *Neto2* male (**d**) and female (**e**) mice during contextual fear acquisition and contextual fear memory retrieval test. Mean +/- 1 standard error is shown. Genotype effect calculated by mixed ANOVA (acquisition) or t-test (context test). P-values surviving multiple testing correction are shown. M=males, F=females, KO=knock out, WT=wild type, US=unconditioned stimulus, Cx ret=context retrieval.

Figure 3. Cued fear conditioning and extinction in *Neto1*$^{-/-}$, *Neto1*$^{+/+}$, *Neto2*$^{-/-}$, and *Neto2*$^{+/+}$ mice, and Morris water maze, displaced and novel object recognition, and spontaneous alternation in *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice. Protocol for investigating fear conditioning and extinction memory (**a**). Cx ret=context retrieval, Cue ret=cue retrieval, Ex ret=extinction retrieval. Percent time freezing for *Neto2* males (**b**) and females (**c**), and *Neto1* males (**d**) and females (**e**). Genotype effect calculated by t-test

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(Cx ret) or mixed ANOVA. Percentage of extinguished mice **(f, g, h** and **i)**. Genotype effect calculated by log rank (mantel-cox) comparison. **(j)** Time spent in quadrants of the Morris water maze during the probe trial. NE=northeast, SE=southeast, SW=southwest, NW=northwest. (T) indicates the quadrant that contained the escape platform during training. Genotype effect calculated by t-test **(k)** Time spent around objects during displaced and novel object recognition tasks. DO=displaced object, NDO=non-displaced object, NO=novel object and FO=familiar object. Genotype effect calculated by Wilcoxon test **(l)** Alternation score in the T-maze task. Genotype effect calculated by t-test. Each dot represents one animal and dashed line in **(l)** indicates chance level. Mean +/- 1 standard error is shown. P-values surviving multiple testing correction are shown. M=males, F=females, KO=knock out, WT=wild type.

Figure 4. *Neto2* **is expressed in the medial prefrontal cortex (mPFC), dorsal and ventral hippocampus (dHpc and vHpc), and amygdala (Amg).** Atlas representation of brain regions analyzed by in situ hybridization: **(a)** mPFC (Cg1=cingulate cortex 1, PL=prelimbic cortex and IL=infralimbic cortex), **(b)** dHpc (CA1, CA3 and DG=dentate gyrus), **(c)** vHpc (vCA1, vCA3 and vDG) and **(d)** Amg (LA=lateral amygdala, BLA=basolateral amygdala, CE=central amygdala and ITCs=intercalated cells) [51]. In situ hybridization (ISH) of *Neto2* in **(e)** Cg1, **(f)** PL and **(g)** IL subregions of mPFC; **(h)** CA1, **(i)** CA3 and **(j)** DG subregions of dHpc; **(k)** vCA1, **(l)** CA3 and **(m)** DG subregions of vHpc and **(n)** LA, **(o)** BLA and **(p)** ITCs sub-regions of Amg. *Neto2* probe specificity was tested using *Neto2* knock out tissue and a sense probe (Figure S3).

Figure 5. *Neto2* **is expressed in both excitatory and inhibitory neurons in fear-related brain regions.** High magnification representative images of *Neto2* (red) and *Gad1* (marker of inhibitory neurons; green) or *Vglut1* (marker of excitatory neurons; green) mRNA expression in **(a)** Cg1, **(b)** PL, and **(c)** IL subregions of mPFC; **(d)** percentage of *Neto2*-expressing cells that also express *Gad1* or *Vglut1* in mPFC. *Neto2, Gad1*, and *Vglut1* mRNA expression in **(e)** CA1, **(f)** CA3, and **(g)** DG

subregions of dHpc; **(h)** percentage of *Neto2*-expressing cells that also express *Gad1* or *Vglut1* in

dHPC. *Neto2, Gad1*, and *Vglut1* mRNA expression in **(i)** vCA1, **(j)** vCA3, and **(k)** vDG subregions of

vHpc; percentage of *Neto2*-expressing cells that also express *Gad1* or *Vglut1* in **(l)** vHPC. *Neto2, Gad1*,

and *Vglut1* mRNA expression in **(m)** LA, **(n)** BLA, and **(o)** ITC subregions of Amg; percentage of

Neto2-expressing cells that also express *Gad1* or *Vglut1* in **(p)** Amg. Cg1=cingulate cortex 1,

PL=prelimbic cortex, IL=infralimbic cortex, DG = dentate gyrus, LA = lateral amygdala,

BLA=basolateral amygdala, and ITCs=intercalated cells.

Figure 6. GLUK2/3 and GLUK5 kainate receptor subunit abundance in lysates and crude

synaptosomes from cerebellum (Cb), ventral hippocampus (vHpc), medial prefrontal cortex

(mPFC), and amygdala (Amg) of *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice. (a) Brain regions dissected for the

immunoblot staining. **(b)** Representative bands from immunoblots of brain lysates from *Neto2*$^{-/-}$ and

Neto2$^{+/+}$ mice using antibodies against NETO2, GLUK2/3, and GLUK5. **(c)** Validation of

synaptosomal enrichment: NETO2 (synaptic marker), synaptophysin (SYP; presynaptic marker), PSD-

95 (postsynaptic marker) and KAR subunits GLUK2/3 and GLUK5 from Cb homogenate (H, n=3) and

synaptosomes (SYN, n=3). For quantification, each lane was first normalized to the b-actin signal and

then to the homogenate level. P-values derived from t-test. **(d)** Representative bands from

synaptosomal immunoblots from *Neto2*$^{-/-}$ and *Neto2*$^{+/+}$ mice using antibodies against NETO2,

GLUK2/3, and GLUK5. **(e)** Ratio of GLUK2/3 and GLUK5 in *Neto2*$^{-/-}$ (Cb, n=7; vHpc, n=7; mPFC,

n=4; AMG, n=6) *vs* WT mice (Cb, n=7; vHpc, n=7; mPFC, n=5; Amg, n=6) calculated from three

replicate immunoblots. Prior to calculating the ratio, protein level from each lane was normalized to b-

actin loading control. The significance of *Neto2* ablation on GLUK2/3 and GLUK5 protein levels

measured using t-test. *p<0.05, **p<0.01, ***p<0.001. For uncropped blots, see Figure S4.

a

Day 1		Day 2
Acquisition	24 hrs	Context retrieval
Context A		Context A

b Acquisition | Cx ret

M *Neto1* WT n=12
M *Neto1* KO n=11

Freezing (%)

Pre-US, Post-US1, Post-US2, Post-US3, Context(1-5)

c Acquisition | Cx ret

F *Neto1* WT n=13
F *Neto1* KO n=9

padj=0.032

Freezing (%)

Pre-US, Post-US1, Post-US2, Post-US3, Context(1-5)

d Acquisition | Cx ret

M *Neto2* WT n=10
M *Neto2* n=10

Freezing (%)

Pre-US, Post-US1, Post-US2, Post-US3, Context(1-5)

e Acquisition | Cx ret

F *Neto2* WT n=10
F *Neto2* KO n=10

Freezing (%)

Pre-US, Post-US1, Post-US2, Post-US3, Context(1-5)

a Cg1 PL IL

b CA1 DG CA3

c vCA3 vCA1 vDG

d LA CE ITCs BLA

e *Neto2* DAPI — Cg1

f *Neto2* DAPI — PL

g *Neto2* DAPI — IL

h *Neto2* DAPI — CA1

i *Neto2* DAPI — CA3

j *Neto2* DAPI — DG

k *Neto2* DAPI — vCA1

l *Neto2* DAPI — vCA3

m *Neto2* DAPI — vDG

n *Neto2* DAPI — LA

o *Neto2* DAPI — BLA

p *Neto2* DAPI — ITCs

50 µm

mPFC

a Neto2 Gad1
 Neto2 Vglut1

b Neto2 Gad1
 Neto2 Vglut1

c Neto2 Gad1
 Neto2 Vglut1

d

Gad1+ *Vglut1+*

% of *Gad1+* or *Vglut1+* cells / *Neto2+* cells

Cg1 PL IL

dHPC

e Neto2 Gad1
 Neto2 Vglut1

f Neto2 Gad1
 Neto2 Vglut1

g Neto2 Gad1
 Neto2 Vglut1

h

% of *Gad1+* or *Vglut1+* cells / *Neto2+* cells

CA1 CA3 DG

vHPC

i Neto2 Gad1
 Neto2 Vglut1

j Neto2 Gad1
 Neto2 Vglut1

k Neto2 Gad1
 Neto2 Vglut1

l

% of *Gad1+* or *Vglut1+* cells / *Neto2+* cells

vCA1 vCA3 vDG

Amg

m Neto2 Gad1
 Neto2 Vglut1

n Neto2 Gad1
 Neto2 Vglut1

o Neto2 Gad1
 Neto2 Vglut1

p

% of *Gad1+* or *Vglut1+* cells / *Neto2+* cells

LA BLA ITCs

20 μm

a

Cerebellum dissection | vHPC dissection | mPFC punches | Amygdala punches

b

	Cerebellum 20 ug	vHPC 20 ug	mPFC 20 ug	Amygdala 20 ug
NETO2				
GLUK2/3				
GLUK5				
b-actin				
	WT KO	WT KO	WT KO	WT KO

c

Cerebellum

NETO2
PSD-95
SYP
b-actin

H   SYN

***
**
*

Normalized protein level
0   1   2   3

Cerebellum

GLUK2/3
GLUK5
b-actin

H   SYN

***
***

Normalized protein level
0   1   2   3

Synaptosomes   Homogenate

d

	Cerebellum 15 ug	vHPC 15 ug	mPFC 3 ug	Amygdala 5 ug
NETO2				
GLUK2/3				
GLUK5				
b-actin				
	WT KO	WT KO	WT KO	WT KO

e

Ratio *Neto2* KO / *Neto2* WT

Cb   vHpc   mPFC   Amg

GLUK2/3
GLUK5