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Supporting Information for

Sex-Specific and Opposed Effects of FKBP51 in Glutamatergic and GABAergic Neurons: Implications for Stress Susceptibility and Resilience

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Supplemental information van Doeselaar et al, 2023: Sex-Specific and Opposed Effects of FKBP51 in Glutamatergic and GABAergic Neurons: Implications for Stress Susceptibility and Resilience

Supplemental methods

Behavioral tests

OF test

The OF test was performed to assess anxiety-like behaviors and general locomotor activity. Mice were placed in an OF arena (50 cm x 50 cm x 50 cm) made out of gray polyvinyl chloride material and could freely explore the arena during a period of 15 minutes. Light conditions were set at 30 lux. After tracking the behaviors, the analyses were performed in time bins of 300 seconds and total distance travelled in the entire OF arena during the full 15 minutes was taken as a measure for general locomotor activity. Other parameters that were measured were total distance travelled in meters, time spent in seconds and number of entries into the inner zone (dimensions: 26 cm x 26 cm) of the OF.

EPM test

Anxiety-like behavior was further assessed using the EPM test. The EPM apparatus consisted of an elevated (50 cm above the ground surface) cross maze with two open arms (30 cm x 5 cm x 0.5 cm) and two closed arms (30 cm x 5 cm x 15 cm). Light conditions were no higher than 10 lux in the closed arms and 20 lux in the open arms. At the start of the test, mice were positioned in the centre of the cross maze with their head directed towards the left closed arm, after which they could freely explore the apparatus during a period of 10 minutes. Open arm distance in meters, time in seconds and number of entries into the open arms were tracked as measures for anxiety-like behavior and were analysed in time bins of 300 seconds.

NOR and SOR

As a measure of memory performance in a neutral, low-stress environment on two different domains, first the NOR (recognition memory) and later the SOR (spatial memory) were executed. The purpose of this test was to investigate the ability of the mice to either discriminate between a novel and a familiar object or between a novel and a familiar location of the object. To create an environment that was as familiar as possible to the mice (a so-called home cage-like set up) and thereby inducing as little environmental novelty stress as possible, objects and mice were placed in enclosed holding cages (35 cm x 30 cm x 18 cm) with bedding material that was mixed with the scent of all animals involved in the experiment. Transparent lids were used in order to record the behavior of the mouse, while light conditions inside the cage were in between 10 and 25 lux. Two separate objects were built out of black and white Lego© blocks, that were unique enough to allow discrimination, but not too distinct that it could create a potential bias based on preference for one specific object. Groups were then evenly divided over 4 different apparati in which testing took place simultaneously. To exclude any chance of bias due to object or location preference, the familiar and novel object or the familiar and novel location were randomised over the different apparati. At the start of the test mice were placed inside the apparatus and could familiarize themselves with the objects or location for a period of 15 minutes. Mice were then taken out of the apparatus and placed back into their home cage that was transported to the test room. After an inter trial interval of 30 minutes, mice were positioned back into the apparatus containing the novel object or novel object location and a 5-minute retrieval period followed. Exploration time for all objects and object locations was scored manually and a discrimination score between novel and familiar object or object location was calculated.

Fear conditioned context retrieval

Stress-related memory performance was explored using a fear conditioned context retrieval test. For this, animals underwent a 2-day protocol, consisting of a fear conditioning phase on day one, succeeded by a context retrieval test on the second day. In the fear conditioning phase, mice were habituated to the conditioning chamber (Bioseb, Pinellas Park, FL, USA) for 1 minute with the chamber lights switched on, which remained on until the end of the test. Subsequently, a series of 5 shock sequences was initiated, which included a 29.5 second waiting time and a 0.5 second footshock (500 ms, 0.7 mA). Each shock sequence, except the last sequence, was followed by a

5-minute inter-trial interval. After the final shock, a 1-minute interval completed the test. Lights and shocks were operated with the commercially available software Packwin v2.0 (Panlab, Barcelona, ES). 24 Hours after fear conditioning, mice were re-exposed to the aversive environmental context. Animals were placed back into the conditioning chamber with lights off. Upon start of the retrieval test lights switched on and animal's freezing behavior was recorded for 5 minutes. In order to examine memory of the aversive environment, total time freezing and latency to the first freeze were analysed.

RNAScope mRNA in situ hybridization

To check whether knockout of *Fkbp5* was exclusively achieved in either forebrain glutamatergic neurons of *Fkbp5*^{Nex} and GABAergic neurons of *Fkbp5*^{Dlx} mice, an RNAScope mRNA *in situ* hybridization was performed on male mice of both conditional KO lines and *Fkbp5*$^{lox/lox}$ controls (3 *Fkbp5*^{Nex} vs. 3 *Fkbp5*$^{lox/lox}$ and 4 *Fkbp5*^{Dlx} vs. 4 *Fkbp5*$^{lox/lox}$). To this end, frozen brains that were stored at -80°C were sectioned at a thickness of 20 µm using a cryostat microtome. Brain sections were collected on Super Frost Slides and saved at -20 °C. Subsequently, *Fkbp5, Vglut1* (glutamatergic neurons) and *Gad1* mRNA (GABAergic neurons) was stained in the relevant tissues with the RNAscope Fluorescent Multiplex Reagent kit (cat. no. 320850, Advanced Cell Diagnostics, Newark, CA, USA), respectively, using the probes Mm-Fkbp5-C1, Mm-Slc17a7-C2 and Mm-Gad1C3. The RNAscope staining procedure was executed according to the manufacturer's protocol and as previously described(1). Images of the dorsal hippocampus and parietal cortex adjacent to the dorsal CA1 were acquired using a ZEISS confocal microscope with the 20x and 40x objectives. Images were taken using identical settings for laser power, detector gain and amplifier onset. *Fkbp5* mRNA puncta within *Gad1* positive cells of *Fkbp5*^{Dlx} vs. *Fkbp5*$^{lox/lox}$ mice and within *Vglut1* positive cells of *Fkbp5*^{Nex} vs. *Fkbp5*$^{lox/lox}$ mice were quantified manually within the ZEISS ZEN 3.2 (blue edition) software. For *Fkbp5*^{Dlx} mice, images of the dorsal hippocampus (region in between dentate gyrus (DG) and CA1) were selected for both visualization and quantification purposes. However, due to the dense nature of glutamatergic neurons in the dorsal CA1, manual quantification of *Fkbp5* mRNA puncta in *Fkbp5*^{Nex} mice and their *Fkbp5*$^{lox/lox}$ controls was performed in parietal cortical regions adjacent to the dorsal CA1. The dorsal CA1 was nevertheless selected to illustrate differences within this condition. Background *Fkbp5* signal was defined as ≤ 3 puncta per cell based on the residual signal observed in full *Fkbp5*$^{-/-}$ mice(2).

Magnetic Resonance Imaging

Sedation was initiated using 2.5% isoflurane. Animals were stereotactically fixated in a prone position on an MR-compatible animal bed where they were kept under inhalational anesthesia with isoflurane (1.5 - 2.5% in pressured air, with a flow of 1.5 l/min). To prevent drying of the eyes,

Bepanthen cream (Bayer, Leverkusen, DE) was applied. Body temperature was assessed using a rectal thermometer and maintained in the range of 36 - 38°C throughout the experiment by means of a warm water silicon pad. Respiration was measured using a pressure sensor placed under the animal's thorax. Depth of isoflurane anesthesia was adjusted to reach a respiration rate of 80 – 120 breaths per minute. MR images were collected on a horizontal BRUKER Biospec 94/20 animal scanner (Bruker BioSpin, Rheinstetten, Germany), operating at 9.4 Tesla and using a transmit/receive cryo-coil with two coil elements. After general adjustments of the system and collection of localizer scans, a 3D T2*-weighted image was acquired using a FLASH sequence with TE=6.25 ms, TR=34.1 ms, flip angle 10°, matrix size 256 x 166 x 205 points, resolution 0.077 mm isotropic, 2 averages, with fat and outer volume suppression. Acquisition time for the 3D was 41 minutes 8 seconds.

Image processing

Images were transferred to NIFTI format using the Python Bru2 function, with voxel size artificially increased by a factor of 10, in order to fully exploit SPM12 in the subsequent analysis. Brain extraction was based on a three-step procedure: First, a rough segmentation using the Hikishima templates(3) with all 5 tissue compartments was performed, including bias correction. Using the resulting tissue probability maps (p-map), an initial first mask was created from grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) compartments and thresholded at ($p\text{-}map_{GM}$+ $pmap_{WM}$ + $p\text{-}map_{CSF}$) > 0.3. In the binarized first mask, holes are filled. The mask was then smoothed with 8mm, and thresholded at > 0.1. Spatially adaptive non-local means denoising (sanlm, CAT12 toolbox, neuro-jena.github.io/cat/) filtering was performed on the bias corrected images from the first segmentation, i.e. on images which were not yet brain extracted. The first mask was then applied on the sanlm-filtered images. In a second segmentation step, a modified Hikishima version was used, in which the inner CSF and skull bones is represented in different probability maps. This separation was necessary as in our T2*-weighted images (different from the typical T1-weighted images, for which Hikishima templates were generated), ventricular CSF shows high image intensities. A second mask was created, based on ($p\text{-}map_{GM}$+ $p\text{-}map_{WM}$) > 0.1 only. Again, holes were filled. The resulting mask was smoothed by 8mm and thresholded > 0.1. In a third and final step, this brain mask was then applied to the original bias corrected and sanlm-filtered images. The brain-masked images were then co-registered to the Hikishima T2-weighted reference image, and the olfactory bulb and the cerebellum were cut out (due to lower signal intensities caused by the geometry of the surface coil). Finally, an SPM12 old segmentation step was done using the GM, WM and inner CSF compartment tissue templates. The resulting tissue probability maps of both genotypes and both sexes for GM and WM were imported to DARTEL and normalized with isotropic voxel size 0.7 mm, to create a study specific template. Flow fields were transformed into jacobian deformation fields for later deformation-based morphometry (DBM) analysis(4) and were smoothed with a Gaussian kernel of 4 mm. Total brain volume (TBV) as well as volume of the individual tissue compartments were determined from the DARTEL imported images (native space), by summation of the tissue probability values in GM, WM and CSF compartments. The anatomical images were also normalized using the DARTEL flow fields, and a mean image was calculated.

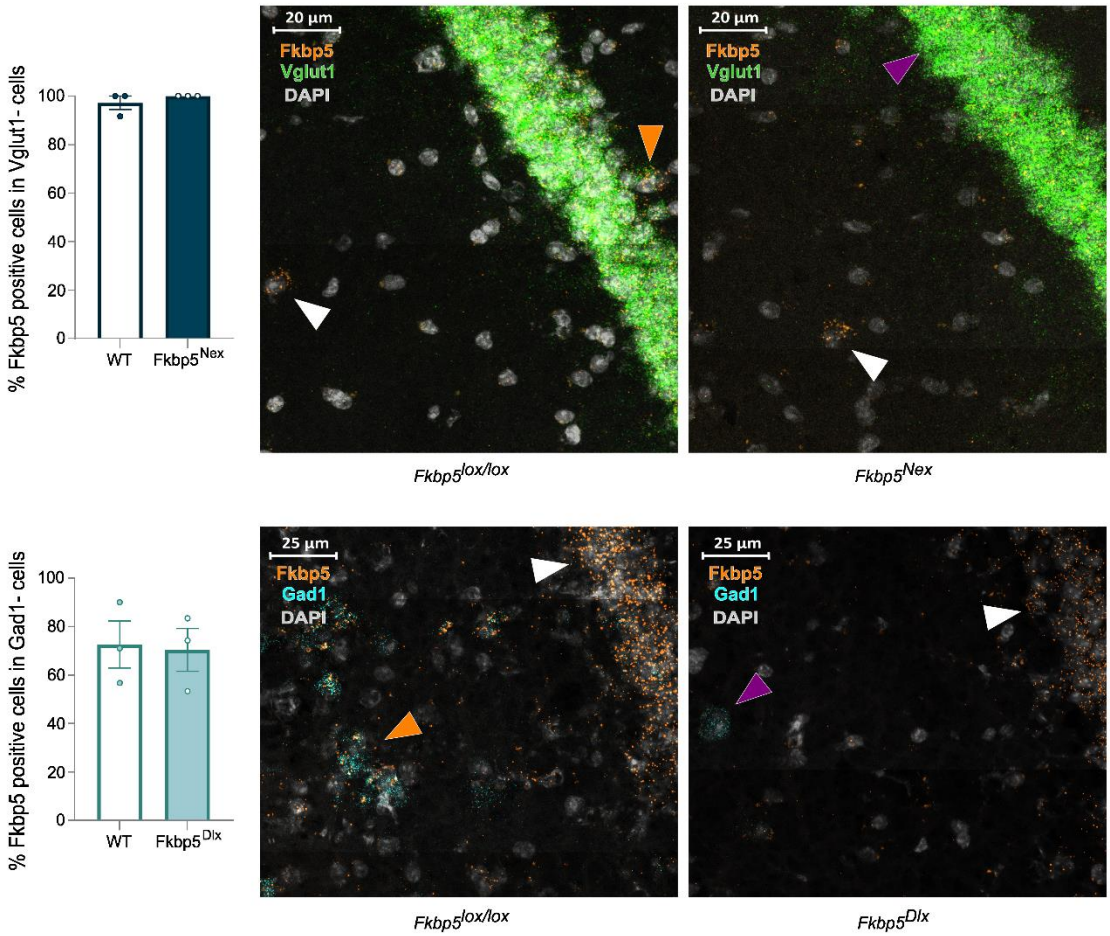

Figure S1. Loss of FKBP51 is restricted to target cell-types in Fkbp5^{Nex} and Fkbp5^{Dlx} mice. Analyses of RNA scope confocal images revealed that there were (A) no differences in % of Fkbp5 positive cells in Vglut1 negative cells of the hippocampus of Fkbp5^{Nex} vs. Fkbp5$^{lox/lox}$ mice. (B) Also, no differences were observed in % of Fkbp5 positive cells in Gad1 negative cells in the hippocampus of Fkbp5^{Dlx} vs. Fkbp5$^{lox/lox}$ mice. White arrow: Fkbp5 mRNA in Vglut1- or Gad1- cell; Orange arrow: Fkbp5 mRNA in Vglut1+ of Gad1+ cell; Purple arrow: Vglut1+ or Gad1+ cell without Fkbp5 mRNA. Error bars represent mean ± S.E.M.

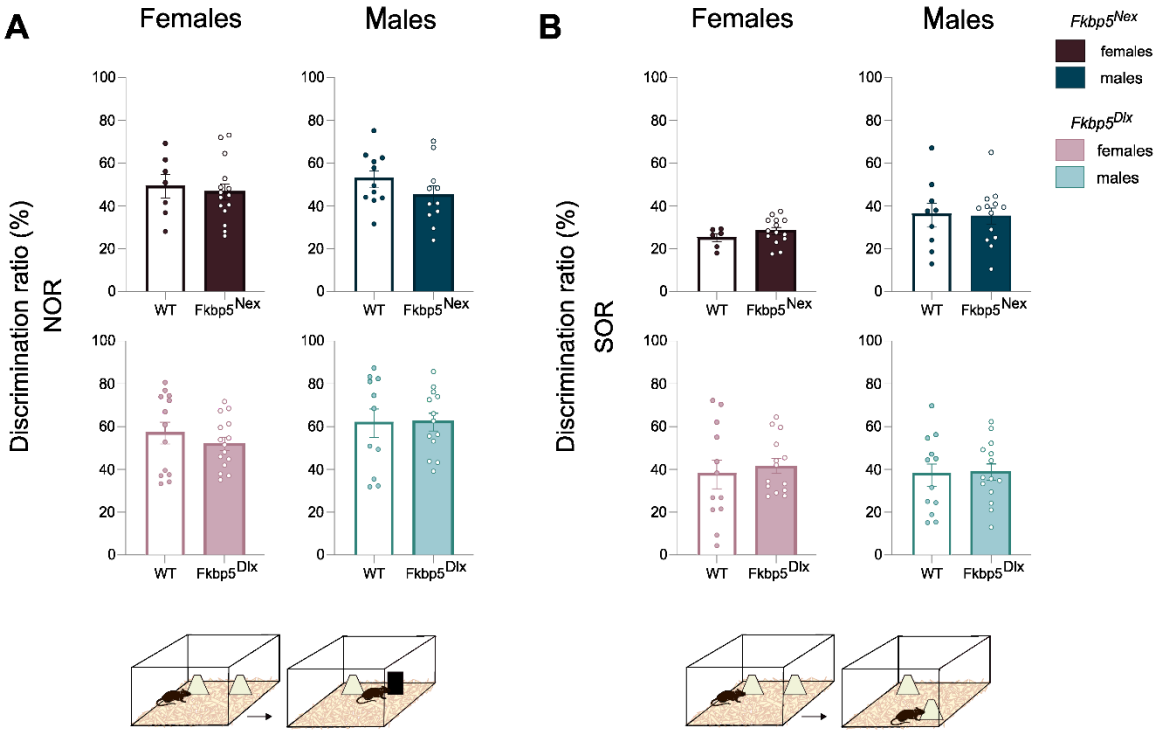

Figure S2. Loss of Fkbp5 in either GABA- or glutamatergic neurons does not affect memory performance in a neutral context. (A) Male, nor female Fkbp5^{Dlx} and Fkbp5^{Nex} mice discriminated between the novel and familiar object in a 5-minute recall session, 20 minutes following a 15-minute exploration of the original object. Like for object recognition memory, (B) spatial location memory was also unaffected by loss of Fkbp5 in either GABA- or glutamatergic neurons in either sex. Error bars represent mean ± S.E.M.

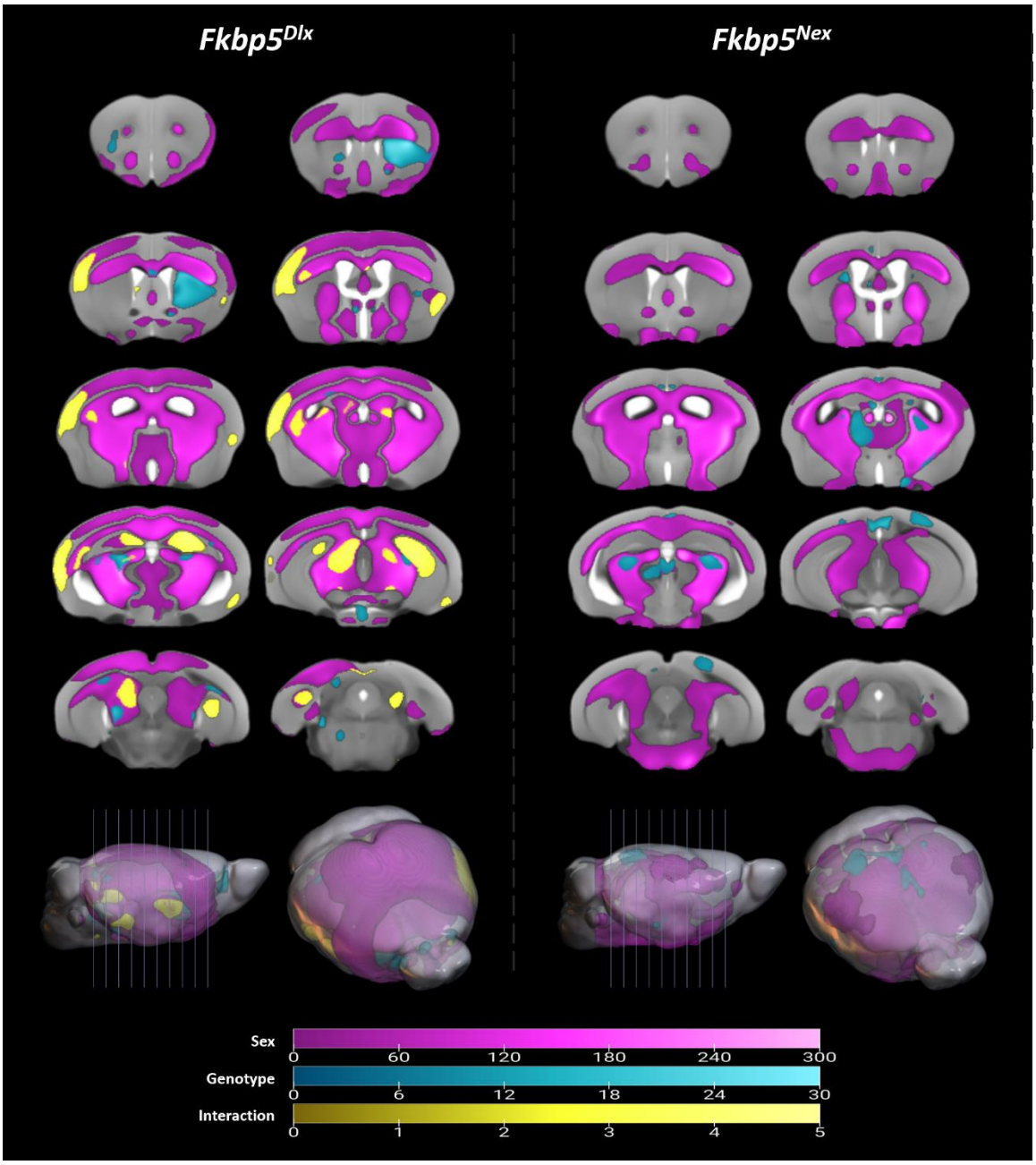

Figure S3. Interaction effects and main effects of genotype and sex in *Fkbp5Dlx* vs. *Fkbp5*$^{lox/lox}$ and *Fkbp5*^{Nex} vs. *Fkbp5*$^{lox/lox}$ mice. Main effects for factors genotype (blue scale), sex (purple scale) and the interaction of sex and genotype (yellow scale). Scales represent Z-scores. Statistically corrected blobs for factor sex are collected at $p_{FWE} < 0.05$, cluster extent > 20 voxel. Statistically significant blobs for factor genotype and interaction of genotype and sex were collected $p_{uncorr} < 0.005$, cluster extent > 20 voxel.

Table S1. Two-way ANCOVA for variables Sex an Genotype and covariate Age for significant physiological and behavioral parameters

Fkbp5 genetic mouseline	Parameter	Sex (sign. main effect)	Genotype (sign. main effect)	Sex * Genotype (sign. interaction)
Fkbp5^{Nex}	Body weight Baseline	F = 98.473 df = 1, 36 p < 0.001	none	F = 4.780 df = 1, 36 p < 0.05
	Relative adrenal weight	F = 84.675 df = 1, 35 p < 0.001	none	F = 2.923 df = 1, 35 p = 0.096
	Elevated plus maze: Distance in open arms	F = 8.538 df = 1, 37 p < 0.01	none	none
	Fear context retrieval: Freezing latency	none	F = 4.720 df = 1, 33 p < 0.05	none
Fkbp5^{Dlx}	Body weight Baseline	F = 4.052 df = 1, 49 p < 0.05	F = 9.367 df = 1, 49 p < 0.01	none
	Relative adrenal weight	F = 109.400 df = 1, 45 p < 0.001	F = 9.800 df = 1, 45 p < 0.01	none
	Elevated Plus Maze: Distance in open arms	none	F = 7.759 df = 1, 48 p < 0.008	none
	Fear context retrieval: Freezing lactency	none	F = 3.106 df = 1, 47 p = 0.085	F = 7.371 df = 1, 47 p < 0.01

SI References

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