Supplementary Information

Association between *SNAP-25* gene polymorphisms and cognition in Autism: functional consequences and potential therapeutic strategies

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Supplementary Methods and Materials

Genotyping

Genomic DNA was isolated from peripheral human blood by phenol-chloroform extraction using standard procedures. SNPs were typed using the TaqMan® SNP Genotyping Assays (Life Technologies) on an ABI PRISM 7000 Sequence Detection System. For rs363039, rs363043, rs363050 and rs3746544 respectively the C_327976_10, C_2488346_10 C_329097_10, and C__27494002_10. Human Pre-Designed Assays (Life Technologies) were used. The restriction enzyme polymorphism rs1051312 was genotyped by DdeI digestion as previously described.[1]

Transfections and luciferase assays

Cell lines and cultures. The SH-SY5Y human neuroblastoma cell line was grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Lonza). The cell line was maintained at 37°C in 5% CO_2. *Plasmid constructions.* A 747 bp fragment, spanning region of SNAP-25 intron 1 and containing the rs363050 polymorphism (A/G), was amplified by PCR. Amplification from 100 ng of genomic DNA from parental or polymorphic allele carriers was carried out in a thermocycler with 1 cycle of 2 minutes denaturation at 95°C, 10 cycles of denaturation at 95°C for 30 seconds, annealing at 55ºC for 30 seconds, and elongation for 30 seconds at 72°C, 15 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 59 °C and 30 seconds elongation at 72 °C, followed by 1 cycle of 5 minutes elongation at 72 °C, using Expand High Fidelity^{PLUS} PCR System (Roche Applied Science). The primers used were the following: forward # 5'- GGA TCC TTA GAC TAG GAT TCA CCA GGC C - 3'; reverse # 5'- GGA TCC CCC AAA GCA GGT GGT AA – 3'; the Bgl II site (underlined) was added to clone the amplified product in the thymidine kinase (TK)-luc/pGL4 basic reporter vector. All restriction enzymes were purchased from NEB (New England

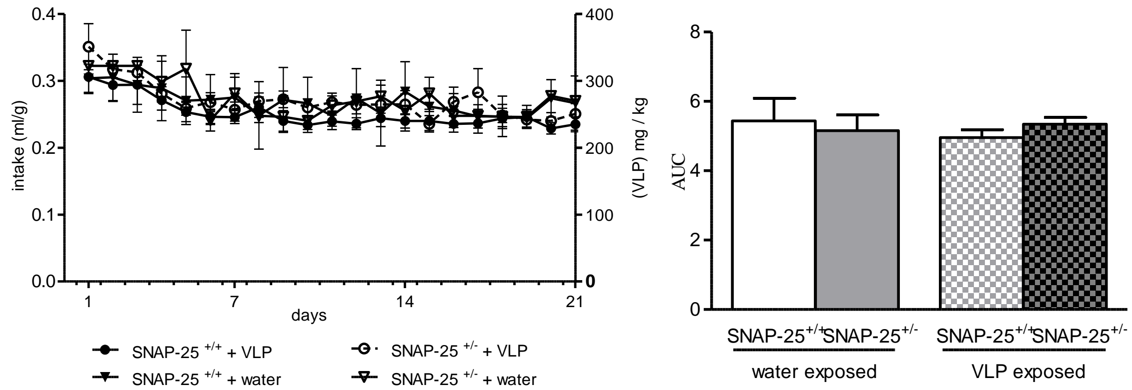
Biolabs). The rs363050A-TK-luc and the rs363050G-TK-luc constructs were obtained by cloning the 747 bp fragment into the Bgl II site (upstream the TK promoter) and into the Bgl II compatible Bam HI site (downstream the luciferase gene; TK-luc-rs363050A and TK-luc-rs363050G) of pTK-luc/pGL4b. The correct orientation and the identity of the inserts were verified by restriction analysis and DNA sequencing. *Transient transfections.*The cells were transiently transfected by means of lipofection using 2 x 10^5 SH-SY5Y cells. Briefly, the cells were plated onto a well of a six-multiwell tissue culture plate (Euroclone) the day before transfection. 100 ng of pRL-TK were mixed with an equimolar amount of the construct of interest and 2 µl of FUGENE HD (Promega). After 20 minutes of incubation at room temperature, the DNA-lipid mixture was added to the cells and incubated for 48 hr, when luciferase activity was measured. The pRL-TK plasmid expresses the Renilla luciferase reporter gene under the control of the thymidine kinase minimal promoter and was cotransfected in each sample to normalize for transfection efficiency.*Firefly luciferase and Renilla luciferase assays***.** Firefly and Renilla luciferase activities were detected by using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instruction in a Berthold L1920 luminometer for 12 s. The transient transfection data were analyzed as previously described.[2]

EEG Surgery*.* Mice were anesthetized with an intraperitoneal injection of 5% chloral hydrate dissolved in saline and given a volume of 10 ml/kg. Four screw electrodes (Bilaney Consultants GMBH, Dusseldorf, Germany) were inserted bilaterally through the skull over cortex (anteroposterior,+2.0–3.0 mm; left–right 2.0 mm from bregma) as previously described[3] according to brain atlas coordinates.[4] A further electrode was placed into the nasal bone as ground. The five electrodes were connected to a pedestal (Bilaney, Dusseldorf, Germany) and fixed with acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy). Animals were allowed a week for recovery from surgery before the experiment. *Procedure.* The signal was

transmitted from the test mouse, through the electrodes, to an amplifier (Bio Amp) and then to a PC which allowed, by means of a software (Chart5, ADInstruments, Castle Hill, Australia) to show the cortical variations of the electric potentials in a graphical elaboration through the monitor. Alterations of mice behaviour were continuously observed during the experiments, through a video camera placed inside the Faraday chamber. EEG traces were analyzed as elsewere described[3] for spike activity. For each EEG recording, the histogram of the maximum positive increments overlapping 20 ms windows was derived. Increments above a threshold determined according to the increments distribution through an unsupervised approach and whose amplitude was greater than twice the background were considered as spikes.

Supplemental Results

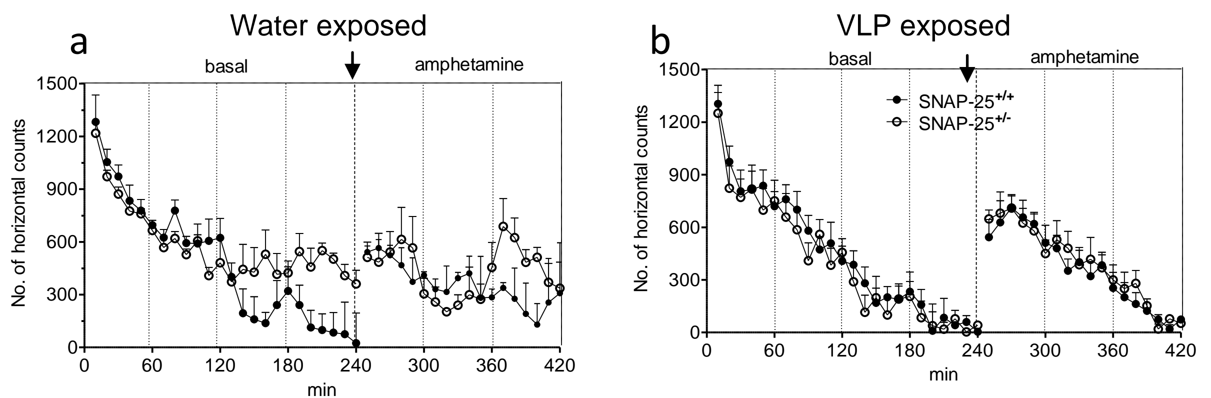
VLP intake. The mean daily intake throughout the exposure is shown in Figure S1 (left) and the corresponding calculated area under the curve (AUC) is shown in the right.The mean intake of VLP corresponded to an amount of about 250-270 mg kg^{-1}. This dosage was found to be effective when acutely given to *SNAP-25*$^{+/-}$ 3-month old mice to reverse behavioral and EEG abnormalities[5]. No difference was found in the mean intake for all the observed groups (two-way repeated measure ANOVA, with treatment as between subjects factor and genotype as within subject factor, treatment x genotype interaction: $F_{(1,36)} = 0.35$, $P = 0.55$; effect of treatment: $F_{(1,36)} = 0.04$, $P = 0.84$; effect of genotype: $F_{(1,36)} = 0.98$, $P = 0.33$).



Supplementary Figure 1. Effect of valproate sodium salt (VLP) on fluid intake. Left panel: Mean (± SEM) daily intake of VLP (1 mg ml^{-1}) or water in *SNAP-25*$^{+/+}$ and *SNAP-25*$^{+/-}$ mice, expressed as fluid intake (ml g^{-1} mouse) (left axis) or amount (mg) of VLP (right axis). Right: area under the curve of fluid intake obtained during exposure (21 days).

Motor activity. The time course of horizontal activity, recorded every 10 min for 4 h (baseline) and after amphetamine injection for 3 h after water or VLP pre-exposure, is reported in Figure S2 Panel a and b. During the first 2 h, both genotypes showed a similar horizontal activity, independently of the treatment they received. However, during the following 2 h (120–240 min), *SNAP-25*$^{+/-}$ water-exposed mice failed to habituate, thus resulting more active than *SNAP-25*$^{+/+}$ littermates. As expected, subcutaneous injection of d-amphetamine (4 mg kg^{-1}) (arrow) increased horizontal activity in *SNAP-25*$^{+/+}$ mice during the first h after treatment (240–300 min), whereas in the following two hours (300–420 min) the stimulant effect decreased. Conversely, d-amphetamine did not appear to exert any effect on *SNAP-25*$^{+/-}$ mice during the first h after treatment, whereas it significantly reduced motor activity during the following hour. A recovery of the motor function was obtained during the last period. In wild-type mice horizontal activity had the same trend in water or VLP exposed animals, while in *SNAP-25*$^{+/-}$ mice VLP pre-exposure rescued the hyperactivity. After amphetamine treatment the trend of motor

activity was comparable in both genotypes.



Supplementary Figure 2 . Effect of valproate sodium salt (VLP) on basal and d-amphetamine-induced motor activity. The time-course of horizontal movements, cumulated every 10-min, recorded 4 h before (basal) and 3 h after amphetamine injection in water-exposed (a) and VLP (b) adolescent *SNAP-25*$^{+/+}$ and *SNAP-25*$^{+/-}$ mice, is shown.

Supplemental References

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