Lentiviral-mediated Let-7d microRNA overexpression in the hippocampus induced anxiolytic- and anti-depressant-like behaviors and impaired dopamine D3 receptor expression in mice

Amine Bahi, Jean-Luc Dreyer

Supplementary Methods

Construction, production of the lentiviral vectors and stereotaxic injection

The let-7d overexpression was achieved by cloning an approximately 450 bp fragment containing the rat let-7d precursor hairpin loop flanked by *BamHI* and *XhoI* restriction sites into pTK431 transfer vector (generous gift from Dr. Tal Kafri, Gene Therapy Center, University of North Carolina at Chapel Hill).

The pTK431 is a self-inactivating HIV-1 vector which contains the entire Tet-off-inducible system, the central polypurine tract (cPPT) and the woodchuck hepatitis virus post-transcriptional regulatory element. It was generated by ligating a *Bgl*II/*Bam*HI DNA fragment containing the tetracycline-regulated transactivator (tTA) (5′) and the tetracycline-inducible promoter (3′) into a *Bam*HI site downstream of a CMV promoter in a self-inactivating HIV-1 vector (Kafri, 2004). The plasmids were then $CsCl_2$-purified, and lentiviruses were produced by the transient calcium phosphate co-transfection of human embryonic kidney 293T (HEK293T) cells with pTK vectors together with pMDG-VSV-G and pΔNRF. Cells were harvested, and viral particles were concentrated from the supernatant by ultracentrifugation. Vectors were resuspended in PBS–BSA and stored at −80°C till use as fully described in our early studies (Bahi et al., 2005). The viral titers were determined by p24 antigen measurements (KPL, USA). For the stereotaxic injections,

the different viral stocks matched for viral particle content were used at approximately 0.2 mg/mL of p24, corresponding to 8-9 × 10^9 IU/mL.

Stereotaxic injection and virus delivery

For stereotaxic injections, the mice were anesthetized with a ketamine/xylazine mixture (100 mg/kg and 10 mg/kg respectively, i.p.) and installed in a stereotaxic frame. The hair from top of the head was removed using an electric razor and a linear skin incision was made, using a scalpel, on the top of the head down the midline to expose the skull. Then, an approximately 1-mm burr hole was drilled in the skull using a hand-held driller at the appropriate coordinates. Using a precision Hamilton micro-syringe, mice were bilaterally infused with a total of 1 µl viral solution (0.25 µL for each site). Before each injection, the syringe was lowered 0.3 mm past the injection site and kept a lower depth for one minute to increase spread of the viral diffusion. Each injection was made over 3 min, and the injector remained in place for an additional 5 min for dispersion before the incision being closed.

Behavioral analyses

Open field (OF) test

In brief, each mouse was individually placed in the corner of a Plexiglass arena (32 × 32 × 20 cm^3), divided into 64 equal squares by black lines, and allowed to freely explore for 20 min. The 16 central squares are regarded as the "center" of the field. Behavioral parameters including the time spent in the center of the arena, the number of fecal boli and the number of line crossings were hand scored and precautions were taken to keep the experimenter blind to condition.

Elevated-plus maze (EPM) test

Briefly, The EPM apparatus consisted of a black wooden maze of two open arms (40 × 6 × 0.2 cm^3) and two closed arms (40 × 6 × 20 cm^3) facing each other and connected by a central platform (6 × 6 cm^2). The maze was elevated 40 cm above the floor. Testing began by placing each mouse in the central square facing an open arm. The number of times a mouse entered fully (whole body) into an open or closed arm (OA and CA respectively) and the time a mouse spent in each arm of the maze were manually recorded for 5 min-period. After each session, the maze was cleaned with a 70% pure ethanol solution to remove odor trails.

Tail suspension test (TST)

In brief, each mouse was suspended 50 cm above the surface of a table, covered with soft cloth, by its tail from a metal rod. The tip of the mouse's tail was fixed on the rod using adhesive Scotch tape. To prevent the tail-climbing behavior, we place hollow plastic cylinders around the base of the tail of the mice. The mice cannot grip onto these cylinders and therefore cannot climb their tails (Can et al. 2012). We have observed that no mice have climbed their tails when using this approach. Latency to immobility and duration of immobility were manually recorded during a test period of 6 min. Immobility was defined when the mouse hung passively and completely motionless.

Forced-swim test (FST)

During the 6 min-test, each mouse was placed in a clear plastic cylinder (18-cm diameter, 25-cm height) containing fresh water at approximately 25°C. The immobility time was defined as the duration for which the mouse floated in an upright position without struggling and making only slight movements to keep its head above the water. At the end

of each session, the mouse was removed from the container and left to dry in a heated enclosure before returned to its home cage.

RNA isolation and real-time qRT-PCR quantification

Frozen samples were placed on dry ice and cut out into small portions (between 50 and 100 mg) before being homogenized in 1 mL of TRIzol® reagent (Life Technologies Corporation, NY, USA) and processed according to the manufacturer's guidelines. RNA pellets were dissolved in nuclease-free water and the concentration of RNA was measured using a NanoDrop 1000 spectrophotometer. The reverse transcription of RNA (4 μg) was performed using SuperScript® III Reverse Transcriptase Kit, containing oligo-dT primers, according to manufacturer's instructions (Life Technologies Corporation, NY, USA). The qRT-PCR primers were as follows: let-7d: 5′-AGG TTG CAT AGT TTT AGG GCA-3′ and 5′-AAG GCA GCA GGT CGT ATA GT-3′; U6 SnRNA: 5'-CTC GCT TCG GCA GCACA-3' and 5'-AAC GCT TCA CGA ATT TGC GT-3'; D3R: 5’-GGG GTG ACT GTC CTG GTC TA-3’ and 5’-TGG.CCC TTA TTG AAA ACT GC-3’; Cyclophilin:5′-GTG AGA AGG GCT TTG GCT AC-3′ and 5′-TTC TCG TCA GGAAAG CGG-3′. The PCR conditions were 95°C for 4 min, followed by 40 cycles of 95°C for 45 sec, and 64°C for 45 sec. The expression levels of let-7d and D3R were normalized with reference to the expression levels of U6 snRNA and cyclophilin respectively, and the fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$).

Statistical analysis

The data representing the effect of let-7d overexpression on anxiety- and depression like behaviors were analyzed using one-way measure of variance (ANOVA). Also, the effects of let-7d overexpression on let-7d and D3R expression levels were analyzed using one-

way ANOVA. The data representing the effects of D3R overexpression and doxycycline on anxiety- and depression like behaviors and on D3R mRNA expression were analyzed using two-way ANOVA with viral-injection (Mock *vs.* D3R) and doxycycline (Water *vs.* DOXY) as the between subject's factor. Simple linear regression (Pearson's) analysis was performed to examine the correlation between let-7d expression with anxiety and depression measures and D3R mRNA expression. Also, in the 2nd set of experiments, the same simple linear regression was performed to examine the correlation between D3R mRNA expression with anxiety and depression measures. A series of post hoc correlations between levels of let-7d and D3R from all subjects and expression of specific anxiety- and depression-like behaviors in all tests were performed using separate linear regressions, in order to provide further insight how changes in let-7d and D3R expression may contribute to alterations in specific anxiety- and depression-like behaviors (Watt et al., 2009). Levene's test was used to inspect the equality or homogeneity of variances of the groups compared. In case of a significant main effect, post hoc comparisons were performed with Bonferroni's test. The criterion for statistical significance was set at $p \leq 0.05$.

Supplementary Results

Pearson's correlations following let-7d overexpression

Using the data of let-7d transcription levels obtained from the Mock- and let-7d-injected mice, a Pearson's analysis was performed to examine the correlation between let-7d transcription levels and parameters of anxiety- and depression-like behaviors. Post hoc linear regressions revealed that differences in let-7d levels within the hippocampus accounted for many of the behavioral alterations exhibited by the mice. In fact, in the OF test, increased let-7d expression was negatively correlated with the number of fecal boli ($F_{(1,22)}$ = 10.132, p = 0.004; **Suppl. Fig. 1A**), but not with the number of line crossings ($F_{(1,22)}$ = 0.019, p = 0.890; **Suppl. Fig. 1B**). In the EPM test, let-7d expression correlated positively with the percentage of time spent into the OA ($F_{(1,22)}$ = 6.904, p = 0.015; **Suppl. Fig. 1C**), and with the percentage of entries into the OA ($F_{(1,22)}$ = 4.721, p = 0.041; **Suppl. Fig. 1D**). In the TST, the results have shown a trend toward a positive correlation between let-7d with the latency to immobility ($F_{(1,22)}$ = 3.410, p = 0.078; **Suppl. Fig. 1E**). Finally, in the FST, increased let-7d expression was positively correlated with climbing duration ($F_{(1,22)}$ = 8.771, p = 0.007; **Suppl. Fig. 1F**).

Post hoc linear regressions revealed that alterations in D3R mRNA expression, following let-7d overexpression, accounted for many of the behavioral changes (**Suppl. Fig. 2**). In fact, in the OF test, D3R mRNA expression correlated positively with the number of fecal boli ($F_{(1,22)}$ = 9.823, p = 0.005; **Suppl. Fig. 2A**), but not with the number of line crossings "spontaneous locomotor activity" ($F_{(1,22)}$ = 0.706, p = 0.410; **Suppl. Fig. 2B**). In the EPM test, D3R expression correlated negatively with the percentage of time spent into the OA ($F_{(1,22)}$ = 14.150, p = 0.001; **Suppl. Fig. 2C**), but not with the number of entries into the

CA ($F_{(1,22)} = 0.061$, $p = 0.808$; **Suppl. Fig. 2D**). In the TST, results also indicated a negative correlation between D3R mRNA with the latency to immobility ($F_{(1,22)} = 7.233$, $p = 0.013$; **Suppl. Fig. 2E**). In the FST, decreased D3R levels were negatively correlated with climbing duration ($F_{(1,22)} = 7.015$, $p = 0.015$; **Suppl. Fig. 2F**).

Anxiety-like behavior following D3R overexpression

The impact of D3R overexpression in the hippocampus on anxiety-like behavior was determined using the OF and the EPM tests respectively and results are depicted in **Suppl. Fig. 3**.

The open field test (OF): The overall activity was estimated by the number of line crossings during a 20-min observation period. The two-way ANOVA revealed that this parameter was not affected by viral-injection as D3R-overexpressing mice showed no difference in the number of line crossings compared with Mock controls ($F_{(1,36)} = 0.623$, $p = 0.435$). Also, doxycycline had no effect on the number of line crossings ($F_{(1,36)} = 0.076$, $p = 0.785$). Therefore, the interaction between the two factors was not found significant ($F_{(1,36)} = 0.036$, $p = 0.851$) indicating no change in overall ambulatory behavior (**Suppl. Fig. 3A**). Interestingly, the analysis of the number of fecal boli using two-way ANOVA revealed a significant effect of viral-injection ($F_{(1,36)} = 6.642$, $p = 0.014$) and doxycycline ($F_{(1,36)} = 9.565$, $p = 0.004$), with a significant interaction ($F_{(1,36)} = 11.225$, $p = 0.002$) (**Suppl. Fig. 3B**). Post hoc evaluations indicated that D3R overexpression increased the number of fecal boli (WTR-Mock *vs.* WTR-D3R, $p = 0.001$). However, when mice had access to doxycycline in the drinking water, no significant difference was found between the two experimental groups (DOX-Mock *vs.* DOX-D3R, $p = 1.000$ and WTR-D3R *vs.* DOX-D3R, $p < 0.001$).

The elevated plus maze test (EPM): as depicted in **Suppl. Fig. 3C**, and consistent with the findings from the OF test, the two-way-ANOVA analysis revealed that D3R-injected mice spent less time the OA (main effect of viral-injection: $F_{(1,36)} = 5.924$, $p = 0.020$). When given doxycycline in drinking water, the time spent into the OA was significantly increased (main effect of doxycycline: $F_{(1,36)} = 5.669$, $p = 0.023$). Interestingly, there was a significant interaction between the two factors ($F_{(1,36)} = 4.743$, $p = 0.036$). Again, the D3R effects were driven by the WTR group, which was significantly different between the viral-injection conditions ($p = 0.015$), whereas the doxycycline group did not differ between Mock- and D3R-injected rats ($p = 1.000$ and WTR-D3R *vs.* DOX-D3R, $p = 0.016$; Bonferroni post hoc test).

Depression-like behavior following D3R overexpression

To investigate the effect of D3R overexpression on depression-like behavior, the TST the FST were performed and results are depicted in **Suppl. Fig. 4**.

The tail suspension test (TST): as depicted in **Suppl. Fig. 4A**, the analysis of the latency to immobility using two-way ANOVA revealed a significant effect of viral-injection ($F_{(1,36)} = 8.045$, $p = 0.007$) and doxycycline ($F_{(1,36)} = 6.608$, $p = 0.014$), with a significant interaction ($F_{(1,36)} = 4.889$, $p = 0.033$). Post hoc evaluations indicated that D3R overexpression decreased the latency only in the WTR group (WTR-Mock *vs.* WTR-D3R, $p = 0.006$). However, when mice had access to doxycycline, no significant difference was found between the two experimental groups (DOX-Mock *vs.* DOX-D3R, $p = 1.000$ and WTR-D3R *vs.* DOX-D3R, $p = 0.010$).

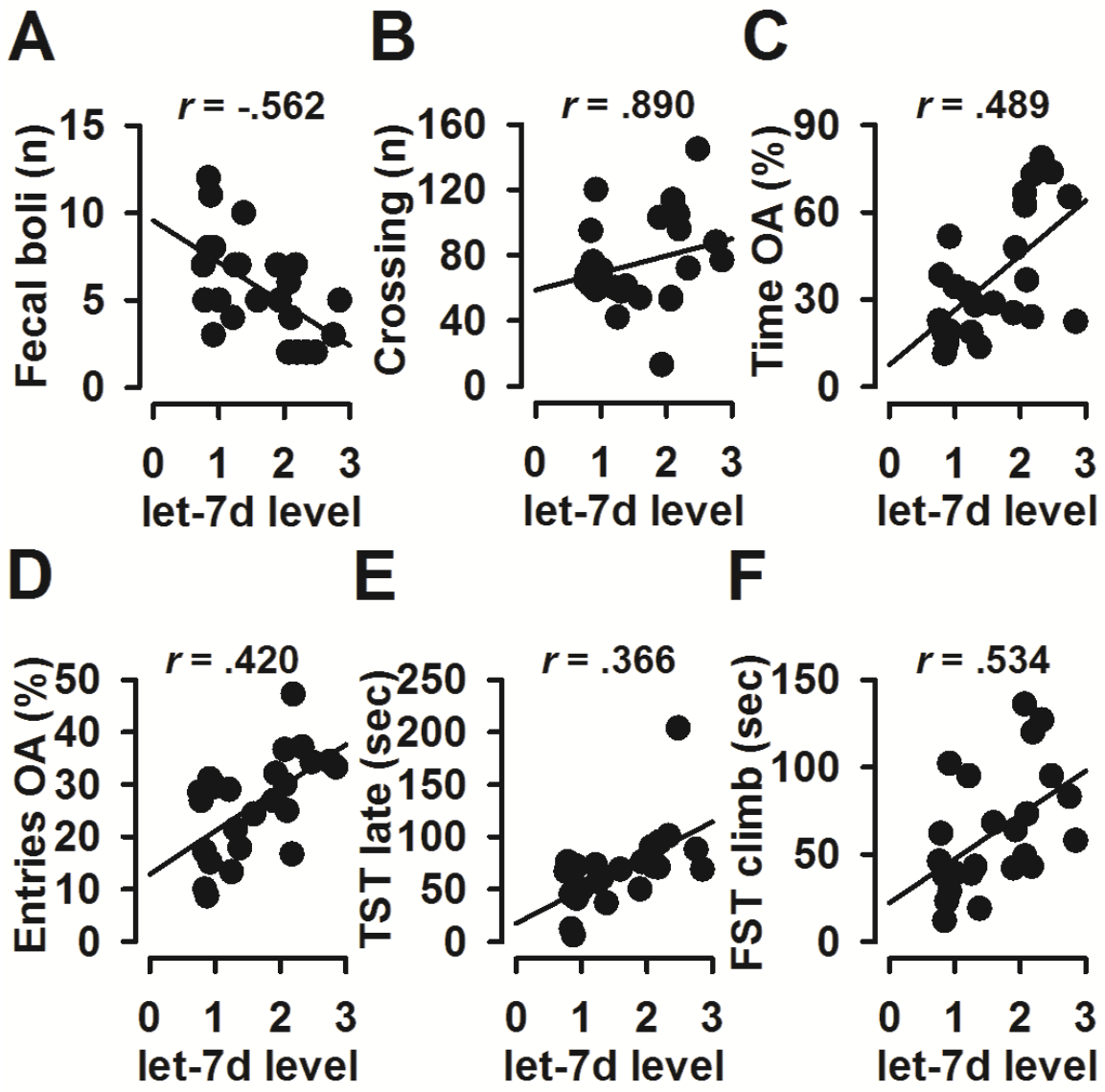
The forced swim test (FST): in this test and as displayed in **Suppl. Fig. 4B**, significant effects of viral-injection ($F_{(1,36)} = 5.942$, $p = 0.020$), and doxycycline ($F_{(1,36)} = 6.168$, $p =$

0.018) on the climbing duration were also found with significant interaction ($F_{(1,36)} = 4.331$, $p = 0.045$) (Data not shown). Pairwise comparisons indicated that the climbing time was significantly shortened after D3R overexpression only in the WTR group ($p = 0.017$). However, there was no significant effect of D3R overexpression on the climbing duration in presence of doxycycline ($p = 1.000$) and (WTR-D3R *vs.* DOX-D3R, $p = 0.016$; Bonferroni post hoc test).

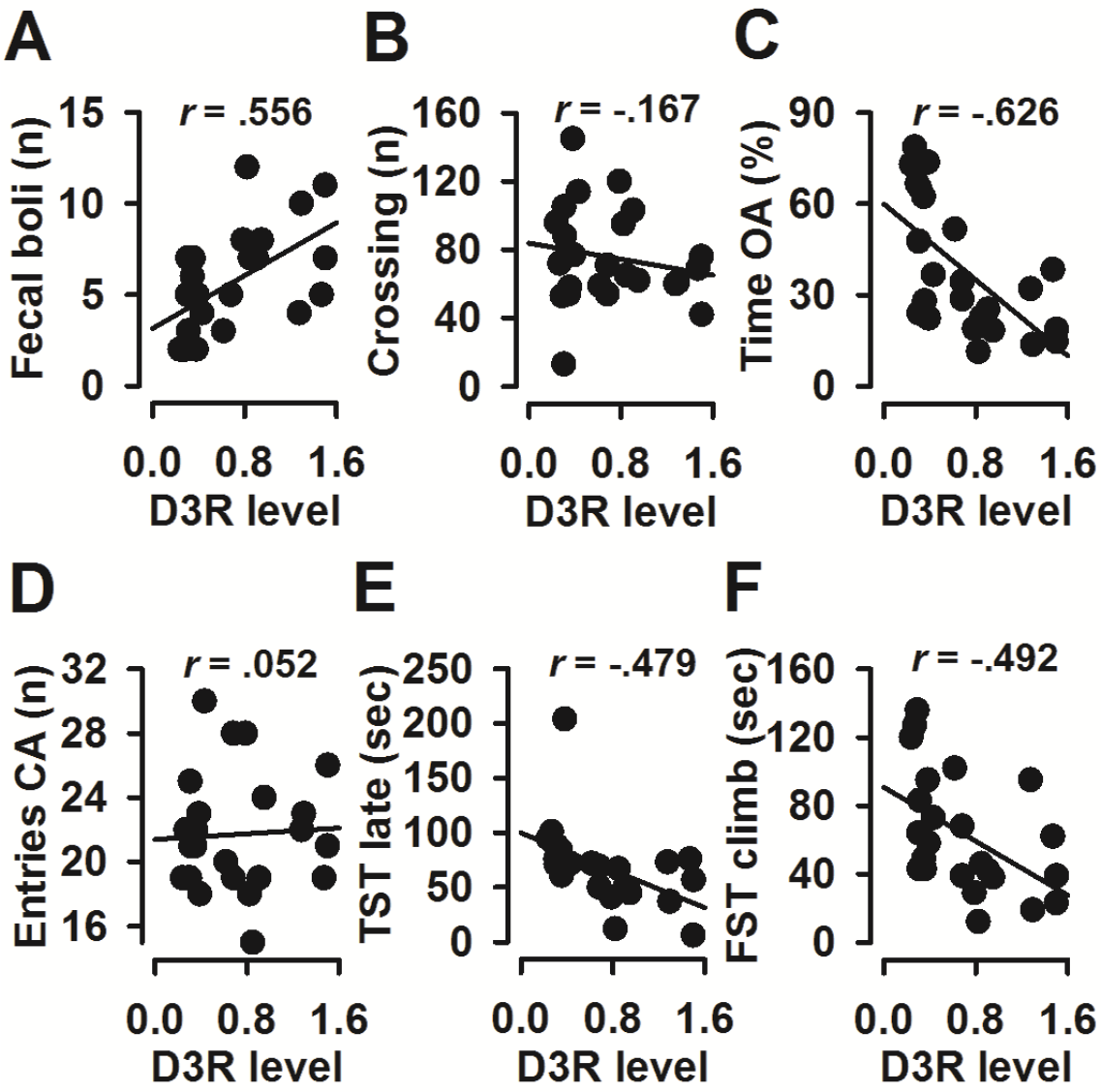
Pearson's correlations following D3R overexpression

Using the data of D3R mRNA levels obtained from the four experimental groups, we performed a Pearson's linear regression analysis to examine the correlation between D3R mRNA levels and anxiety- and depression-like behaviors. The scatter plots are provided in **Suppl. Fig. 5**. In the OF test, D3R correlated positively with the number of fecal boli ($F_{(1,38)} = 9.409$, $p = 0.004$; **Suppl. Fig. 5A**), but not with the number of line crossings ($F_{(1,38)} = 0.090$, $p = 0.766$; **Suppl. Fig. 5B**). In the EPM test, D3R expression correlated negatively with the percentage of time spent into the OA ($F_{(1,38)} = 12.956$, $p = 0.001$; **Suppl. Fig. 5C**), and with the percentage of entries into the OA ($F_{(1,38)} = 13.832$, $p = 0.001$; **Suppl. Fig. 5D**). In the TST, results also indicated a negative correlation between D3R mRNA with the latency to immobility ($F_{(1,38)} = 18.914$, $p < 0.001$; **Suppl. Fig. 5E**). In the FST, decreased D3R levels were negatively correlated climbing duration ($F_{(1,38)} = 10.980$, $p = 0.002$; **Suppl. Fig. 5F**).

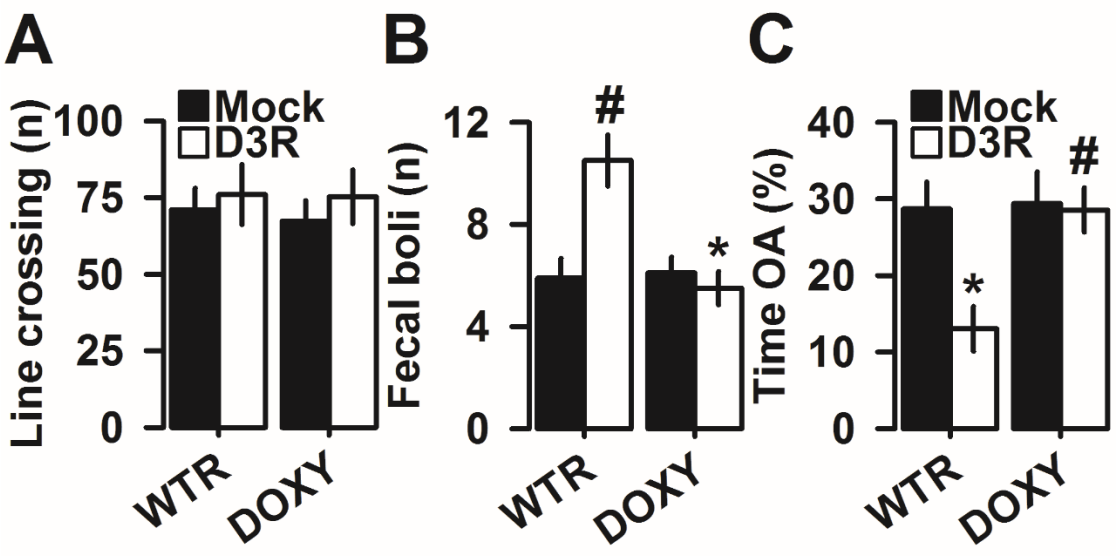
Supplementary Figures



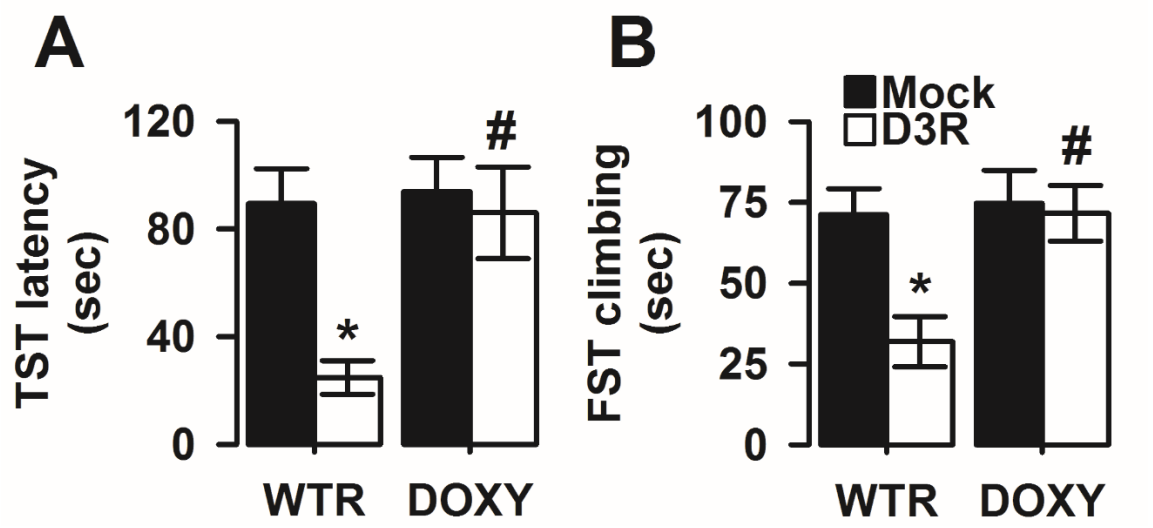
Suppl. Fig. 1. Pearson's correlations between let-7d expression and anxiety- and depression like behaviors. The data represent simple scatter regression between let-7d levels with the **A)** number of fecal boli, and **B)** number of line crossings of the OF test, **C)** percentage of time spent, and **D)** percentage of entries into the OA of the EPM test. **E)** latency to immobility in the TST, and **F)** duration of climbing in the FST.



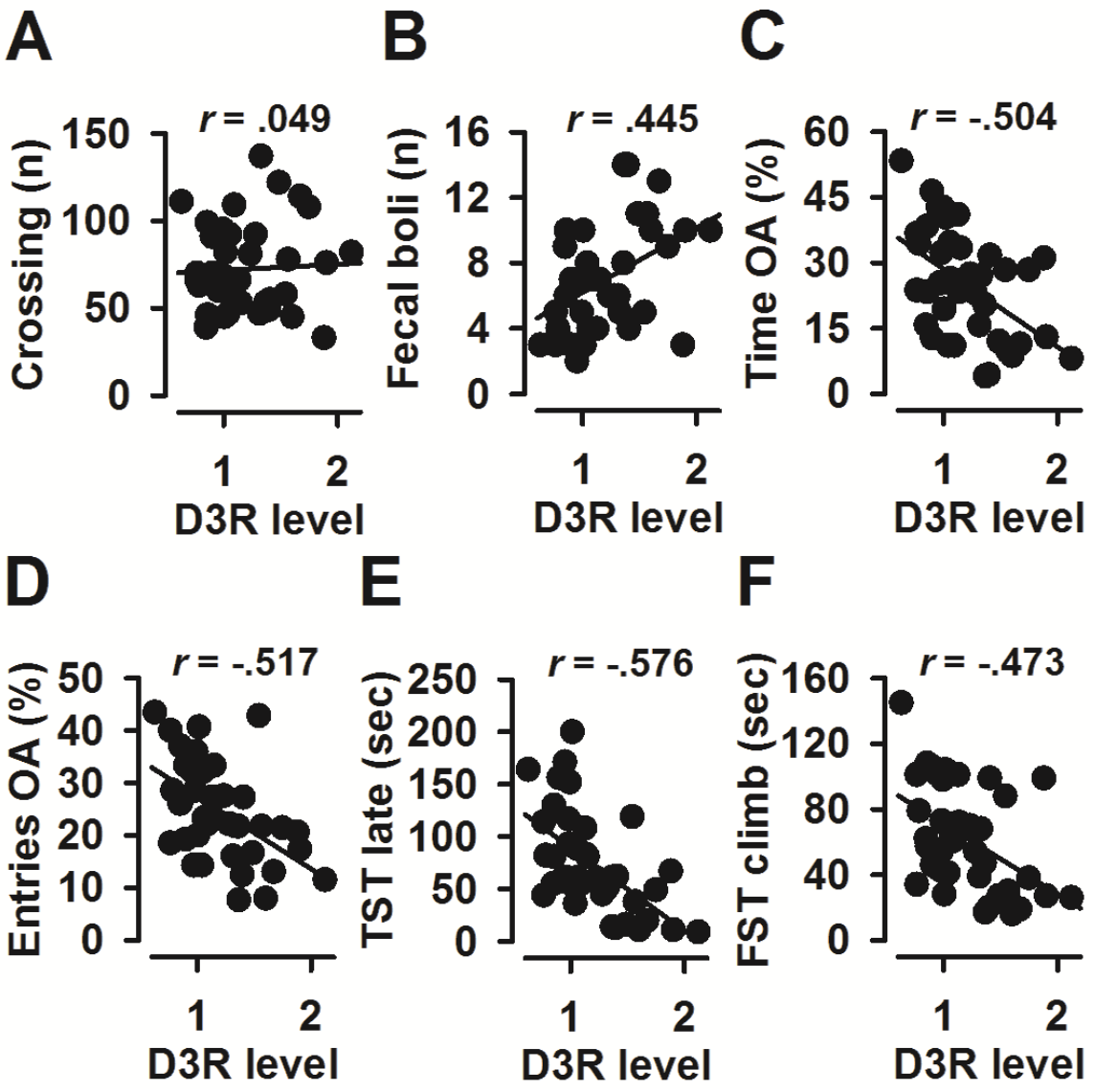
Suppl. Fig. 2. Pearson's correlations between D3R mRNA expression and anxiety- and depression like behaviors. The data represent simple scatter regression between D3R levels with the **A)** number of fecal boli, and **B)** number of line crossings of the OF test, **C)** percentage of time spent into the OA, and **D)** number of entries into the CA of the EPM test. **E)** latency to immobility in the TST, and **F)** duration of climbing in the FST.



Suppl. Fig. 3. Anxiety-like behaviors in Mock- and D3R-injected mice in the OF, and EPM tests with access to water (WTR) or doxycycline (DOX). For the OF test, the data are expressed as mean ± SEM for the **A)** number of line crossings, and **B)** number of fecal boli. For the EPM test, the data are expressed as mean ± SEM for the **D)** percent of time spent in the OA. *$p < 0.05$ indicate significant differences between Mock and D3R; #$p < 0.05$ indicate significant differences between WTR and DOX. For all the groups n = 10.



Suppl. Fig. 4. Depression like behaviors in Mock- and D3R-injected mice in the TST and FST with access to water (WTR) or doxycycline (DOX). For the TST, the data are expressed as mean ± SEM for the **A)** latency to immobility. For the FST, the data are expressed as mean ± SEM for the **B)** duration of climbing. *$p < 0.05$ indicate significant differences between Mock and D3R; #$p < 0.05$ indicate significant differences between WTR and DOX. For all the groups n = 10.



Suppl. Fig. 5. Pearson's correlations between D3R mRNA expression in Mock- and D3R-injected mice and anxiety- and depression like behaviors. The data represent simple scatter regression between D3R levels with the **A)** number of line crossings, and **B)** number of fecal boli of the OF test, percentage of **C)** time spent, and **D)** entries into the OA of the EPM test, **E)** duration of latency in the TST, and **F)** duration of climbing in the FST.

	let-7d overexpression
Open field test	
Crossing (n)	$F_{(1,22)} = 2.865$, $p = 0.105$
Fecal boli (n)	$F_{(1,22)} = 0.685$, $p = 0.417$
Time in center (sec)	$F_{(1,22)} = 0.194$, $p = 0.664$
Elevated plus maze test	
Time OA (%)	$F_{(1,22)} = 2.279$, $p = 0.102$
Entries OA (n)	$F_{(1,22)} = 0.762$, $p = 0.392$
Entries OA (%)	$F_{(1,22)} = 0.057$, $p = 0.814$
Entries CA (n)	$F_{(1,22)} = 0.238$, $p = 0.631$
Tail suspension test	
Immobility latency (sec)	$F_{(1,22)} = 0.195$, $p = 0.663$
Immobility duration (sec)	$F_{(1,22)} = 0.231$, $p = 0.636$
Forced swim test	
Immobility duration (sec)	$F_{(1,22)} = 1.416$, $p = 0.247$
Swimming duration (sec)	$F_{(1,22)} = 0.002$, $p = 0.968$
Climbing duration (sec)	$F_{(1,22)} = 0.862$, $p = 0.363$

Suppl. Table 1. Levene's tests for equality of variances for measures of anxiety- and depression-like behaviors following let-7d overexpression.

	D3R overexpression
Open field test	
Crossing (n)	$F_{(3,36)} = 0.853$, $p = 0.474$
Fecal boli (n)	$F_{(3,36)} = 0.294$, $p = 0.829$
Time in center (sec)	$F_{(3,36)} = 1.219$, $p = 0.317$
Elevated plus maze test	
Time OA (%)	$F_{(3,36)} = 1.166$, $p = 0.336$
Entries OA (n)	$F_{(3,36)} = 1.081$, $p = 0.369$
Entries OA (%)	$F_{(3,36)} = 0.688$, $p = 0.565$
Entries CA (n)	$F_{(3,36)} = 0.061$, $p = 0.980$
Tail suspension test	
Immobility latency (sec)	$F_{(3,36)} = 2.619$, $p = 0.066$
Immobility duration (sec)	$F_{(3,36)} = 0.568$, $p = 0.640$
Forced swim test	
Immobility duration (sec)	$F_{(3,36)} = 0.276$, $p = 0.842$
Swimming duration (sec)	$F_{(3,36)} = 2.157$, $p = 0.110$
Climbing duration (sec)	$F_{(3,36)} = 0.666$, $p = 0.579$

Suppl. Table 2. Levene's tests for equality of variances for measures of anxiety- and depression-like behaviors following D3R overexpression.