Nurturing nature : testing the three-hit hypothesis of schizophrenia

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Chapter 4

Testing the three-hit hypothesis of schizophrenia: ACTH hyper-reactivity and schizophrenia-like endophenotypes co-precipitate in genetically-susceptible rats following early-life adversity and post-weaning social isolation experiences

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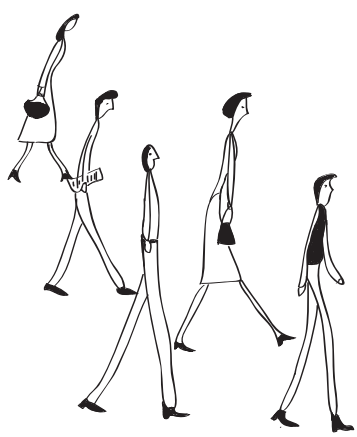

In preparation.

Abstract

It has been postulated that common variance in dopamine related genes producing dopamine dysregulation is causally involved in schizophrenia pathophysiology, if combined with adverse early postnatal and prepubertal environmental factors.
The present study was designed to test this "*three-hit* or *cumulative* stress" hypothesis of schizophrenia by examining these environmental interactions in rats with dopaminergic hyper-reactivity.
Methods
We used as a first hit the genetic predisposition for dopaminergic hyper-reactivity of the apomorphine susceptible (APO-SUS) rat line. Poor maternal care and post-weaning isolation rearing served as second and third hit to brain development and maturation of the APO-SUS rats, respectively. Animals were assessed on dopamine sensitivity (APO-induced gnawing), sensorimotor-gating (pre-pulse inhibition of acoustic startle, i.e. PPI), short-term memory (T-maze spontaneous alternation) and stress response (hormone responses to a conditioned emotional stressor).
Results
1. APO-SUS rats did not differ from outbred Wistar Hannover (WH) control rats in PPI and short-term spatial memory, but displayed attenuated acoustic startle and impaired contextual fear acquisition. Exposure of the APO-SUS rats to a conditioned emotional stressor revealed blunted prolactin and enhanced ACTH release, but no difference from WH in the CORT response. APO-SUS individuals' gnawing and PPI performance, in contrast to WH, were resistant to acute exogenous corticosterone (CORT), while they had increased expression of mineralocorticoid receptors in the hippocampus.
2. Adult APO-SUS rats having experienced poor maternal care as pups in the form of Low Licking and Grooming (LG), developed a baseline PPI-deficit, but showed enhanced short-term memory. Their stress-induced CORT secretion was enhanced together with an enhanced prolactin release and a dramatically enhanced ACTH release. High LG offspring on the contrary displayed enhanced PPI that was reduced only after a supraphysiological dose of corticosterone or an apomorphine challenge.
3. Additional isolation rearing abolished entirely baseline PPI and impaired their short-term memory in the Low LG APO-SUS offspring , while High LG offspring were protected from post-weaning adversity.
Conclusion
A severe schizophrenia-like phenotype precipitates if genetically predisposed rats are exposed to early-life adversity and a chronic psycho-social stressor initiated at juvenility. Genetically selected "reactive" dopaminergic alleles amplify the individual's vulnerability to schizophrenia–like phenotypes after cumulative exposure to stressors.

1. Introduction

Psychotic disorders, like schizophrenia, are driven by genetic and environmental risk factors [1]. Common gene variants (e.g. COMT, DRD2, Akt1) predisposing for altered dopamine neurotransmission are strong candidates in the list of schizophrenia-susceptibility genes [2, 3]. Although heritability is often emphasized, the onset of the

psychotic symptoms in schizophrenia is associated with environmental risk factors such as early-life adversity, birth or upbringing in an unfavorable social environment and drug abuse [1]. These environmental factors are perceived by the organism as stressors and alter the hypothalamic – pituitary – adrenal (HPA) axis activity. There is evidence suggesting a link between altered HPA-axis activity, striatal hyper-dopaminergic activity and psychotic symptoms [4-9]. In the present study we tested the "*three-hit* hypothesis" of schizophrenia which postulates that in genetically susceptible individuals the cumulative exposure to adverse early-life experience and pre-pubertal social environment may lead to a complex schizophrenia-like phenotype [10-14].

We did use the apomorphine-susceptible rat line (APO-SUS). This rat line was selected from Wistar rats based on the increased stereotypic gnawing response to the dopaminergic agonist apomorphine (APO) [15]. These APO-SUS rats showed several schizophrenia-like abnormalities ranging from behavioral (including sensorimotor gating deficits) to endocrine and immune alterations [15-18]. In order to establish the role of the genotype (Hit 1), we first investigated the phenotype of APO-SUS and used their paternal common outbred Wistar rat strain as control. Rats were assessed on developmental markers (body weight, neonate stress response, eye-opening), dopamine sensitivity (apomorphine-induced gnawing; i.e. APO-gnawing), sensorimotor-gating (pre-pulse inhibition of acoustic startle; i.e. PPI), short-term memory (T-maze spontaneous alternation), and stress response (conditioned response to contextual fear).

Second, since it was shown previously that the APO-gnawing of the adult APO-SUS rats was reduced after crossfostering with APO-unsusceptible (APO-UNSUS) dams [19], we hypothesized that APO-SUS dams might show reduced maternal care behaviors (Hit 2) like licking and grooming (LG). We divided the APO-SUS rats in groups that had received high, medium or low amounts of maternal care the first postnatal week. Next the outcome of these three different early-life experiences was investigated in the adult.

Third, we asked the question if an unfavorable post-weaning social environment could amplify the behavioral alterations caused by maternal care in APO-SUS offspring. We expected a more severe phenotype since isolation rearing alone already can induce schizophrenia-like neuroanatomical, neurochemical and behavioral disruption in common outbred rats [20-24]. All together the data showed that early-life adversity enhanced vulnerability of genetically-susceptible individuals to a later psycho-social stressor resulting in a severe schizophrenia-like phenotype.

2. Materials and Methods

2.1 Animals

Wild-type Wistar-Hannover (WH) and Wistar-APO-SUS rats (obtained from Taconic Europe, Horst, Ejby, Denmark) were used in this study. Upon arrival males and females (F0 generation) were housed in our animal facility in groups of 3 in Type IV cages (L60 x W38 x H20 cm), and used for

breeding after a habituation period of at least one month. Rats were housed under a 11:13 h light/ dark cycle (lights on at 08.30 h). Food and water were available ad libitum. Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC.

2.2 Chemicals

Injections were prepared and preserved on low temperature (4°C). Injections were given subcutaneously (SC) in a volume of 1 ml/kg. Apomorphine (APO; APO-HCL: Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands) was dissolved in MilliQ (0.02% ascorbic acid). Corticosterone (CORT; 45mg Cort-HBC containing 66.7mg/g CORT; Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands) was dissolved in MilliQ (0.02% ascorbic acid). The dissolvent served as vehicle (VEH).

2.3 Pharmacogenetic selection for dopamine susceptibility

A detailed description of the original development of the APO-SUS line in Radbound University Nijmegen can be found elsewhere [15, 16]. Briefly, all males and females of a generation were submitted to a 45min APO-gnawing test (see below) and litters were ranked for average APO-gnawing counts. Out of the highest gnawing APO-SUS litters, the highest gnawing males and females were selected for breeding of the next generation. For each generation brother-sister pairing was prevented. The same procedure was repeated for more than 16 generations, and after this APO-SUS rats were just bred with APO-SUS rats without the repetition of the APO-gnawing test. The APO-SUS rats used in our experiments were rederived from the original APO-SUS population by Taconic Europe (Horst, Ejby, Denmark). The rederivation was successful (I.E.M. de Jong unpublished data). All rats (both WH and APO-SUS) used in our studies, went through the APO-gnawing test anyway to ensure their difference in APO-gnawing.

2.4 General Breeding

Two or three females of the F1 generation were housed together for at least a week and then mated with a male. After 10 days, the females were housed individually (Type III cages with wire lid; L42.5 x W26.6 x H18.5 cm; containing sawdust and two sheets of paper towels for nest material). We checked for litters daily at 19:30h starting from 20 days after the start of breeding. If litters were present, the day of birth was defined as pnd 0 for that litter. On the day after parturition, pnd 1, each litter was culled to 8-10 healthy pups (males:females = 1:1). From pnd 1 to 10, cages were not cleaned and from pnd 11, the cages were changed weekly.

2.5 Maternal behavior observations

The maternal behavior of each dam was observed and scored for five-60min periods per day during the first 7 pnds using a procedure described before [25, 26]. Observations were performed at three periods during the light phase (at 10:00, 13:30 & 17:00h) and two periods during the dark phase (07:30 and 19:30h; under red light). The behavior of each mother was scored every 3min (20 observations per period, 100 observations per day). For our exact protocol the reader is referred to our previous publications [27, 28].

We namely scored the following maternal behaviors: pup retrieval, maternal contact, licking and grooming (LG), passive nursing posture, away from nest, nest building, burying, arched-back nursing [(passive) low arch/ blanket nursing, (active) low arch, middle arch, high arch].

We considered as (overall) passive nursing (PN) the sum of the passive nursing posture and the (passive) low arch back nursing scores. The other three nursing postures (active low arch, middle arch, high arch were considered (overall) active nursing (AN). Non-maternal care behaviors of the dam were also recorded: eating, drinking, chasing tail, self-grooming, digging, and sleeping. Litter conditions were also noticed: split litter, buried pups. We analyzed the percentage of observations in which the dam displayed each behavior. Note that some behavioral categories were not mutually exclusive. In the result section, we report frequencies (as % of observations) of AN, LG, PN, AWAY, SG. We also the report the variation of these behaviors (;measured as the standard deviation of their frequency).

2.6 Naturally occurring maternal environment

Large cohorts of APO-SUS females of F1 (n=16) and F2 (n=20) generation were bred and after parturition were characterized for maternal care as described above. Dams were sorted according to the LG average scores into groups as described before [26, 29]: < one SD below the mean of the whole group (Low LG), between one SD below and one SD above the mean of the whole group (Med LG), and > one SD above the mean of the whole group (High LG). For detailed information of the LG distribution the reader is referred to the supplementary materials & methods.

2.7 Post-weaning housing [Socials, Isolates (isolation rearing), Breeders, Adult isolates (isolation housing)]

On pnd 21, the pups were weaned from their dams; males and females were separated. Three males (socials) of the same litter were housed together in Type IV cage from pnd 21 until testing. One male of the same litter (isolates) was isolated and placed individually in Type III cage from pnd 21 until testing. This is the typical isolation rearing procedure [30]. To disentangle also the effect of adult isolation housing from isolation rearing, we also tested socially reared adult animals just after a 10-day period in social isolation in Type III cages (adult isolates). Finally, we also tested the effect of breeding after a 10-day period in breeding with 2 females (breeders). In all groups, post-weaning cleaning of the cages happened once weekly.

2.8 Experimental design (Fig. S2)

2.8.1 Experiment I - Genetic susceptibility (Hit 1): In order to explore if APO-SUS rats are phenotypicaly different from the WH, we, first, described their differences in developmental parameters (body weight, eye-opening and neonate stress endocrine response) then we explored their phenotypic differences in APO-gnawing, sensorimotor gating (Acoustic Startle & PPI), in short-term spatial memory (T-maze spontaneous alternation) and in the conditioned emotional response (freezing response, and measurement of plasma prolactin, ACTH, CORT levels). Further, we described the effect of CORT on APO- gnawing and sensorimotor gating. The results of this experiment are presented in Figures 1, 2, 3, S3, S4 & Table S1.

2.8.2 Experiment II - Genotype-dependent differences in maternal care: The objective was to investigate if the APO-SUS dams express an altered or reduced maternal care compared to the WH. Results of this experiment are presented in Figures 4 & S5.

2.8.3 Experiment III - interaction of genetic susceptibility with early-life stress (Hit 1 & 2): We tested the hypothesis that APO-SUS individuals with the lowest maternal care history (i.e. Low LG) display deficits in development (body weight, eye-opening and neonate stress endocrine response) and

in behavioral/ endocrine responses (APO-gnawing, sensorimotor gating, short-term memory, conditioned emotional response), as compared to the ones with higher maternal care history (i.e. High LG). We further explored the role of CORT in the development of sensorimotor gating deficits caused by early-life stress. The results of this experiment are presented in Figures 5, 6, 7, S5 & Table S2.

2.8.4 Experiment IV - Interaction of genetic susceptibility and post-weaning social environment (Hit 1 & 3): We investigated the outcome of the post weaning housing for APO-SUS individuals in development (body weight) and in behavior (APO-gnawing, sensorimotor gating, short-term memory). The results of this experiment are presented in Figure S6 & Table S3.

2.8.5 Experiment V – Interaction of susceptibility with both early-life stress and post-weaning social environment (Hit 1, 2 & 3): We tested the hypothesis that the APO-SUS individuals from poor maternal care litters will be more vulnerable to isolation rearing. We measured deficits in development (body weight) and in behavior (APO-gnawing, sensorimotor gating, short-term memory). The results of this experiment are presented in Figure 8 & Table S4.

2.9 Developmental parameters

2.9.1 Body weight

2.9.1.1 Body weight before weaning (pnd 1-21) was measured with an electronic precision scale (MXX-2001, Denver Instrument, Göttingen Germany; readability 0.1 g, linearity 0.2 kg).

2.9.1.2 Body weight after weaning was measured with an electronic precision scale (Access C 13 AB, Precia Molen, Breda, The Netherlands; readability 1 g, linearity 3 kg) during the weekly cage cleaning.

2.9.2 Neonatal endocrine stress response (pnd 5). We determined the HPA-axis responsiveness to a mild stressor at 17:00h on pnd 5. Pups were removed from their nest sacrificed immediately by decapitation or placed individually in new clean cages (Type III, which were divided in compartments of 18 x 20 x 14 cm, containing fresh sawdust bedding). Novelty exposure was carried out in a separate room, the "novelty exposure" room, under similar environmental conditions as the housing room. The cages were placed on heating pads (33–38 °C) to maintain the body temperature of the pups. After 30min in the novel environment, the pups were sacrificed. Trunk blood from all pups was collected and adrenals were dissected, snap frozen in isopentane (surrounded by dry ice) and stored at -80°C until used for Western blotting.

2.9.3 Eye opening. Eyes of both males and females were examined daily at 11:00h from pnd 11. Any degree of eyelid separation in any of the 8 pups of a litter was scored as a positive eye opening for the litter. Pups were not removed from the nest during the observation so that the litters were not disturbed.

2.10 Behavior

2.10.1 APO-gnawing. Rats given psychomimetic drugs often exhibit loco-motor hyperactivity, and at higher doses they might exhibit stereotyped/ perseverative behaviors [31-33]. In order to assess perseverative behavior in our rat populations, we measured the behavioral response to an injection of a large dose of APO using the so-called gnawing box [15, 32].

Apparatus. The gnawing box was slightly modified from the box originally described by Ljungberg and Ungerstedt [32]. It consisted of a Perspex hole-board (L69 x W69 x H25 cm) with a central cubicle (L25 x W25 x H25 cm). The board contained 32 holes (diameter approx. 3cm), each of

which was surrounded by five concentric ridges. A microphone was placed underneath the central cubicle to allow registration of sounds. Stereotypic gnawing on the ridges surrounding the holes produces a characteristic sound that was detected by the microphone, fed into the computer and scored as a gnawing count [15, 19].
Procedure. The procedure followed for the APO-test was described before [15]. Briefly, after 60min of habituation period in the room of the gnawing box with food and water ad libitum, the rat was given a SC APO injection (dose: 1.5mg/kg). Immediately after injection the rat was put in the gnawing box (facing the front right corner) and measuring of gnawing lasted 45min. After the test, the rat was transferred back to its home cage.
Testing conditions.
Basal conditions (1st time): Rats were weighed in the housing room and transferred in a Type III cage to the testing room containing the gnawing box.
Acute CORT condition (2nd time -/+ CORT): Some of the rats were tested in exact the same way for a 2nd time at least one month after the 1st time of APO injection. However, other rats, which were tested also for a 2nd time, were pretreated with CORT (SC, dose: 3mg/kg) one hour prior the test in the beginning of the habituation period.

2.10.2 Prepulse Inhibition (PPI) of Acoustic Startle. Apparatus. PPI measurements were performed in four startle chambers (SR-LAB, San Diego Instruments, San Diego, CA) consisting of a Plexiglas tube (diameter 8.7 cm, length 20.5 cm) attached on top of a piezoelectric accelerometer platform, which detected and transduced the movements of the rat. A speaker above the tube presented the acoustic pulses. Calibration of all of the four chambers was done with a calibration device and protocol provided by the manufacturer and adjustments of the chamber speakers were done daily with the help of decibel-meter (; dB[A] scaling was used).
PPI protocol. The PPI protocol was based on previously described rat protocols [34] and specifically on the protocol used before with APO-SUS rats [17]. Rats were placed individually in the apparatus and the 17min testing protocol started. First, 5min of acclimatization were given with a background noise of 70 dB. Second, the PPI protocol started and consisted of 3 parts. The protocol started with a startle block of 6 pulse alone trials and ended with a startle block of 5 pulse alone trials. In pulse alone trials we used a pulse of 120 dB[A] for 40 ms. The main (middle) part of the protocol consisted of 39 trials: 10 pulse alone trials, 20 prepulse-pulse trials (5 trials for each of the four different prepulse intensities; prepulse intensities: 72, 74, 78, 86 dB[A] for 20 ms), 5 prepulse alone (86 dB[A] was only used) and 4 no-stimulus/background trials. These 39 trials were given in pseudorandom order; preventing two identical trials following each other and ensuring that the interval between two consecutive trials was different (mean intertrial interval duration 15 sec). The prepulse to pulse onset duration was stable at 100 ms.
Testing conditions.
Basal conditions: Rats tested in baseline conditions were transferred in their home cage into the testing room with the startle chambers and allowed a 45min habituation period in the room. After PPI testing, they were weighted and then returned to their respective cages.

Acute CORT condition: Animals when tested after drug administrations (SC injections) were weighted before PPI testing, because injection volume was calculated using the body weight. Rats were weighted in the housing room and transferred individually in Type III cages to the testing

room to habituate for 45min. Rats were SC injected with VEH, CORT (at dose 3 mg/kg) and APO (at dose 0.5 mg/kg). The three littermates were randomly subjected to one of the three injections. After injection, rats were put back in their cages for 5min, then into the startle chambers and the PPI protocol started. Note that injections happened 10min before PPI testing (counting additional 5min of acclimatization period as part of the PPI protocol). VEH and APO injections were the control conditions for the CORT injection since VEH was expected not to disrupt PPI (negative control) and APO was expected to disrupt it (positive control).

Measurements. The startle response after each trial was calculated by the software and the unit of measurement is Volts. The software was set to gather 1000 samples per sec for a sampling period of 100msec after the onset of the pulse. A Vmax was determined and Vaverage (Vavg) was calculated for the whole 100msec period. We used Vmax as more accurate since Vavg depends on the duration of the sampling period.

Startle reactivity and Startle Habituation: The initial startle response is considered too variable and was discarded according to previously described protocols [34]. The average of pulse alone trials No 2-6 was used as the startle reactivity. To access habituation of acoustic startle, we compared the startle responses in the initial part (1st startle block) of the testing protocol with the other two startle blocks (middle pulse alone trials, last five trials).

PPI: PPI % (for each prepulse intensity) was calculated as: 100% x (Avg. Middle 10 Pulse alone trials – Avg. PPIx trials)/ Middle 10 Pulse alone trials. The average of PPI of the four different prepulse intensities was used as a measure of overall PPI.

2.10.3 Spontaneous alternation in the T-maze

Apparatus. A Plexiglas T-maze with transparent walls and a black floor was used. The T-maze was divided in three arms: start (L75xW12xH20 cm), left (L32xW12xH20 cm) and right (L32xW12xH20 cm). Two sliding doors permitted to close the entrance of the left and right arm respectively. A metal grid cover was additionally used so that the rats were not able to escape from the maze. The T-maze was placed in the housing room in such a way that the amount of luminescence was the same in the right and the left arm of the T-maze (15 LUX).

Spontaneous alternation protocol. The T-maze was used to investigate if the different rats would spontaneously alternate. An experimental session consisted of a sample trial and a choice trail. In the sample trial, the animal was placed in the start-arm of the T-maze and allowed to explore the whole maze. Once the animal entered one of the targeted arms, the sliding door was closed preventing the animal leaving this arm. The arm entered (left or right) was registered, as well as the latency of the entry. Head dips which were made in the arms before an entry were also registered. A maximum of 90sec was given for an entry. After 90sec (if an entry was not made) or after 20sec after the time of entry, the animal was taken out of the maze and put into a type III cage. The number of defecations and urinations were registered and the maze was cleaned with a 10% alcohol solution and dried with a tissue. Directly after the choice trial followed. The rat was returned to the start-arm, with the two arm-sliding doors open again, and allowed to explore again. After 90sec (if an entry was not made) or after 10 sec after the time of entry, the animal was taken out of the maze and put back to its home cage. The arm entered (if any) in the second trial was registered. Two sessions were conducted per day (at 12:00 & 16:00h) for 3 days in a row.

Measurements. The percentage of sessions (% spontaneous alternation) that a rat alternated in the choice trial was used as an output parameter.

2.10.4 Contextual fear conditioning. Fear conditioning in rats has been widely used to study fear formation, recall and extinction [35, 36].
Apparatus. The fear conditioning box (L40xW40xH50 cm) was located in a room with similar environmental conditions as the housing room. The walls of the box were made of black Plexiglas. The floor of the box consisted of stainless steel rods, connected to a shock generator. The box was cleaned with a 10% ethanol solution before rats were placed inside. A video camera placed 20 cm above the box allowed each subject's behavior to be monitored as well as recorded digitally by a computer.
Procedure. Acquisition: Rats were individually transported in a Type III cage from the housing room to an adjacent room containing the fear conditioning set up. The rat was placed in the shock box. After 2min, one electric foot shock (0.6 mA, 2 sec) was given and 2min later, the rat returned to its home cage. Re-exposure: 24h later, the same procedure was repeated however without delivery of the foot shock.
Measurements. Behavior of the rat was recorded by the camera during acquisition and re-exposure. An observer unaware of treatment conditions scored the videotapes using special software (Observer 9.0 XT, Noldus, Wageningen, The Netherlands). Behavior was classified as: (1) freezing (lack of all body movement except that necessary for breathing), (2) scanning (lack of body movement but swaying of the head and breathing), (3) rearing (animal is taking a new position while standing on his hind legs) or (4) default (other).
The factor "time" had three levels: during acquisition: (1) 2min after shock, during re-exposure: (2) first 2min and (3) last 2min. No rat showed more than 10% freezing behavior before the shock, which we had set as exclusion criterion.

2.11 Conditioned emotional response (pnd 180, 188; before and after fear conditioning)
Basal blood samples by tail incision have been taken one week before the experiment. After the re-exposure to the fearful context (fear conditioning box) and the behavioral recordings lasting 4min, rats were kept in a cage in a room next to the housing room. Blood samples were also taken 4, 10, 15, 30, 120min after the onset of the stressor (re-exposure to the fearful context). The tail incision method used has been described before [37].
Some rats that did not go through the fear conditioning experiment were sacrificed by decapitation at basal conditions. During decapitation, hippocampi were dissected, rapidly frozen on dry ice and stored at -80oC until used for Western blotting.

2.12 Tissue measurements
2.12.1 Blood samples handling. Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes, were kept on ice and later centrifuged for 15min at 13000 rpm at 4°C. Plasma was transferred to clean 1.5 ml microcentrifuge tubes. All plasma samples were stored frozen at 20 °C until hormone determination.
2.12.2 ACTH (pg/ml) was measured by radioimmunoassay (MP Biomedicals, LLC, NY, USA; sensitivity 10 pg/ml, intra-assay variation 4.1%, interassay variation 4.4%). Samples were determined in a 50% dilution, starting with 25µl blood plasma. All samples were analyzed in one assay to exclude inter-assay variation.
2.12.3 CORT(ng/ml) was measured by radioimmunoassay (MP Biomedicals, LLC, NY, USA;

sensitivity 1.25 ng/ml, intra-assay variation, 4.4%, interassay variation 6.5%;). Concentrations were determined in duplicate from an extended standard curve (0, 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 ng CORT/ml), since we noted that the lower boundary provided by the kit was not sensitive enough to measure basal plasma concentrations. All samples were analyzed in one assay to exclude inter-assay variation.

2.12.4 Prolactin(ng/ml) was measured by a specific competitive Elisa. Briefly, 96 well cell culture plates (Nunc Maxisorb Immuno Plates; Nunc A/S, Roskilde, Denmark) were coated with capture antibody (100µl of 1:200 Affinity purified Donkey anti Rabbit IgG; Jackson ImmunoResearch Laboratories Inc) and incubated overnight at 40C. Following washing, non-specific binding to the wells was blocked by incubation with assay buffer (200µl: 0.05 M Tris.Cl buffer pH 7.5 containing 1% Bovine serum albumin (BSA) and 0.1% bovine γ globulin) for a minimum of 1h. After washing assay buffer was added to each well (90µl) followed by standard (10µl NIDDK-Rat PRL-RP-3 diluted from 200ng/ml to 0.8ng/ml) or sample including quality controls (10µl). All standards and samples were added in duplicate. Rat anti-prolactin antiserum (50µl; 1:35.000 NIDDK- anti rat Prolactin-RIA-9) and biotinylated rat prolactin(50µl;1:100,000 NIDDK- rat PRL-I-6) were then added and the plates were incubated overnight at 40C. Detection of biotinylated prolactin was by the addition of streptavidin-HRP (100µl; GE Healthcare UK, Little Chalfont, UK) for a minimum of 30min followed by TMB peroxidase substrate (100µl; KPL, Gaithersburg, MD 20878, USA). Color was allowed to develop for a maximum of 10min then the reaction stopped by the addition of 6% phosphoric acid. The Plates were read at 450nm and the results calculated using AssayZap (Biosoft, Cambridge, UK).

2.12.5 Tyrosine hydroxylase (TH), mineralocorticoid receptor (MR) & glucocortocoid receptor (GR) protein levels. Western blotting was performed according to a previously described protocol [27]. The reader is referred to supplementary materials & methods for details.

2.13 Statistical analysis

Data are presented as mean ± SEM and were analyzed by one-way, one-way repeated measures or two-way analysis of variance (ANOVA) with the significance level was set at $p< 0.05$. Where appropriate, simple and interaction main effects were investigated further with subsequent post-hoc comparisons (by Tukey test or student t-tests). For the ACTH and CORT response curve, we could calculate the AUC using Prism GraphPad software. The statistical analysis was adjusted for non-equivalent groups when needed. The initial analysis of pups' measurements included sex as a factor; once it was determined that sex was not a significant factor, data from males and females were pooled. When data from different generations were used together, we have performed separate analyses on each generation. If the different analyses showed the same main effects, the data was pooled.

3. Results

3.1 Experiment I - Genetic susceptibility (Hit 1)

3.1.1 Developmental parameters

3.1.1.1 Body weight (Table S1). From pnd 11, the weight of APO-SUS was significantly lower than WH and this reduced body weight persisted until adulthood. We followed body weight, on a weekly basis, up to pnd 179 and confirmed this genotype effect.

3.1.1.2 Neonatal response to novelty-stress (pnd 5; Fig. S3). ACTH (Fig. S3A): Two-way ANOVA revealed only a main effect of novelty-stress ($F_{1,63}$=7.64; p=0.008), but not of rat genotype or their interaction. Only APO-SUS pups, responded with a significant increase of ACTH towards 30min of novelty (p=0.003).

CORT (Fig. S3B): Two-way ANOVA revealed main effects of rat genotype ($F_{1,63}$=15.65; p=0.001) and of novelty-stress ($F_{1,63}$=24.24; p<0.001), but not of their interaction. Both APO-SUS and WH rats responded with an increase of CORT to 30min of novelty (p=0.001). The basal and stress induced CORT levels of APO-SUS pups were lower than the ones of WH (p=0.010 and p=0.004 respectively).

3.1.1.3 Adrenal TH protein levels (Fig. S3C). One-way ANOVA revealed an effect of rat genotype ($F_{1,63}$=16.54; p=0.001). TH levels were significantly higher in the APO-SUS.

3.1.1.4 Eye-Opening (Fig. S3D). One-way ANOVA revealed an effect of rat genotype ($F_{1,97}$=41.67; p=0.001). APO-SUS rats' eyes open one day later than the WH.

3.1.2 Behavior/ Endocrine measurements

3.1.2.1 Basal APO-gnawing. On average, there was, as expected, a dramatic rat genotype effect in gnawing counts (Fig. 1A); APO-SUS displayed 20-fold higher levels of gnawing than WH ($F_{1,349}$= 2075.162; p<0.001). For both rat genotypes there was not a significant effect of time within the light cycle that the testing happened (Fig. 1B), but the genotype effect was significant in all time points (p<0.001). Within a 45min testing session (Fig. S4), rats of both genotypes showed an increase of gnawing (WH: $F_{44,5148}$= 106.897; p<0.001, APO-SUS: $F_{44,10208}$= 15.462; p<0.001), but for the APO-SUS this increase was dramatic ($F_{1,350}$= 2087.260; p<0.001). APO-SUS and WH rats were different already from the 4thminute of testing (p=0.003) and they showed different gnawing responses over the whole testing period (p<0.001).

3.1.2.2 Basal sensorimotor gating. Acoustic Startle (Fig. 1C): There was a significant effect of startle block within the protocol on acoustic startle for both rat genotypes reflecting habituation ($F_{2,108}$=23.005; p<0.001/WH: p<0.001, APO-SUS: p<0.001). However, the APO-SUS startled overall less than the WH during the whole protocol ($F_{1,55}$=244.117; p<0.001/p<0.001 for all startle blocks).

PPI (Fig. 1D): There was a significant effect of increasing prepulse intensity on PPI in both rat genotypes ($F_{3,162}$=112.350; p<0.001/WH: p<0.001, APO-SUS: p<0.001). This

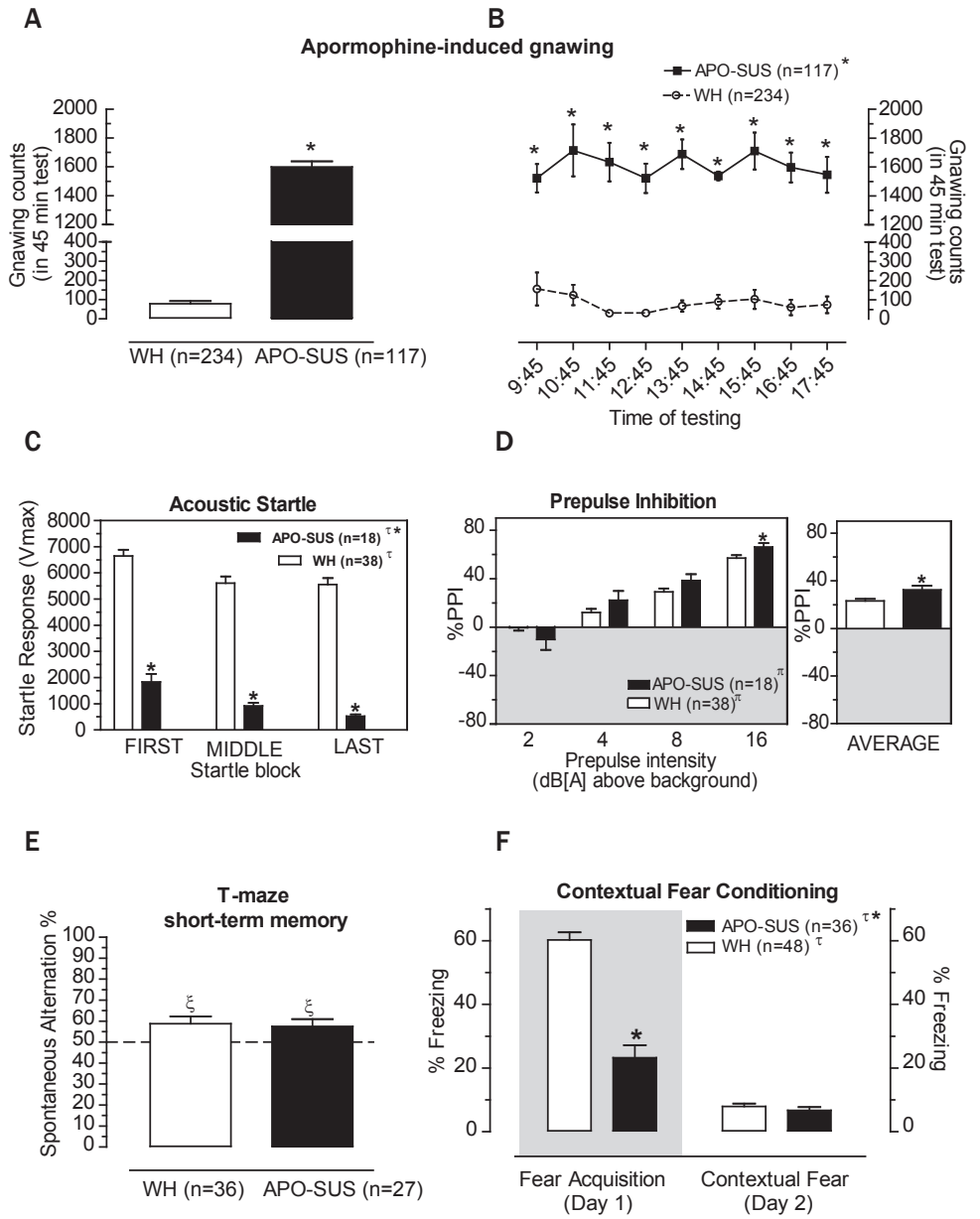

Figure 1. Behavior: Total apomorphine-induced gnawing counts during a 45min observation period (A); Total gnawing counts during a 45 min observation period as a function of the time within the light cycle that the experiment was performed (B); Acoustic startle (C), Prepulse inhibition (D), T-maze short-term memory (E), 24h-Contextual Freezing (F) of apomorphine susceptible rats (APO-SUS) and common Wistar Hannover rats (WH). Left panel D show PPI expressed per prepulse intensity level, whereas right panel D show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. * vs. corresponding values of WH, τ denotes startle block effect, π denotes prepulse intensity effect, ξ vs. 50% (chance level). The exact number of rats used is indicated in the different panels.

effect interacts with the rat genotype effect ($F_{3,162}$=112.350; p=0.023). Further analysis revealed that the APO-SUS displayed higher PPI than the WH at the highest prepulse intensity 16dB[A] over background (p=0.039) and overall ($F_{1,55}$=7.540; p=0.008) compared to WH.

3.1.2.3 T-maze short-term memory (Fig. 1E). There was not a difference in % spontaneous alternation between APO-SUS and WH, but both groups performed over the 50% chance level (APO-SUS: p=0.034, WH: p=0.012).

3.1.2.4 Conditioned emotional response.

Behavioral (Fig. 1F): Repeated measures one-way ANOVA revealed a main effect of time ($F_{2,164}$=136.175; p<0.001/ p<0.001 for both rat genotypes), rat genotype ($F_{1,83}$=14.52; p=0.001), and their interaction ($F_{2,164}$=71.73; p<0.001). For the post-shock 2min period, further analysis revealed that the APO-SUS froze less than the WH during the acquisition of fear (p<0.001). For the first 2min of re-exposure, there was not a difference in freezing between the two rat genotypes and for the last 2min of re-exposure (data not shown) APO-SUS froze more than the WH (p=0.003).

Endocrine: ACTH response (data not shown): Repeated measures one-way ANOVA revealed a main effect of time ($F_{4,216}$=57.51; p<0.001/ p<0.001 for both rat genotypes) and of the interaction of time and rat genotype ($F_{4,216}$=8.59; p<0.001). There was no rat genotype difference in baseline ACTH. However, APO-SUS rats displayed higher ACTH levels than WH at all time points after the onset of the stressor (4-10-15min: p<0.001, 30min: p=0.007). ACTH AUC (Fig. 2A): APO-SUS displayed greater overall ACTH output than the WH ($F_{1,83}$=18.25; p<0.001).

Prolactin release (data not shown): Repeated measures one-way ANOVA revealed a main effect of time ($F_{4,216}$=13.04; p<0.001/ p<0.001 for both rat genotypes) and rat genotype ($F_{1,55}$=25.78; p<0.001). Except for 30min after stress, APO-SUS rats displayed lower prolactin levels than WH including baseline (baseline-4min: p<0.001, 10min: p=0.021, 15min p=0.013).

CORT response (data not shown): Repeated measures one-way ANOVA revealed a main effect of time ($F_{5,270}$=391.793; p<0.001/ p<0.001 for both rat genotypes), and the interaction of time and rat genotype ($F_{5,270}$=7.13; p<0.001). At baseline and 120min after stress, APO-SUS displayed slightly lower but significantly different CORT plasma levels than WH (p=0.034 & p=0.017 respectively). However, at 15min after stress they displayed higher levels than WH (p=0.001). CORT AUC (Fig. 2A): There was no difference in the total CORT output between the rat genotypes.

MR & GR protein levels. Hippocampus MR and GR levels (Fig. 2B): One-way ANOVA revealed an effect of rat genotype in the hippocampal levels of MR protein ($F_{1,69}$=2.26; p=0.020) with APO-SUS rats having higher levels than the WH rats. Pituitary GR levels (data not shown): One-way ANOVA did not reveal an effect of rat genotype.

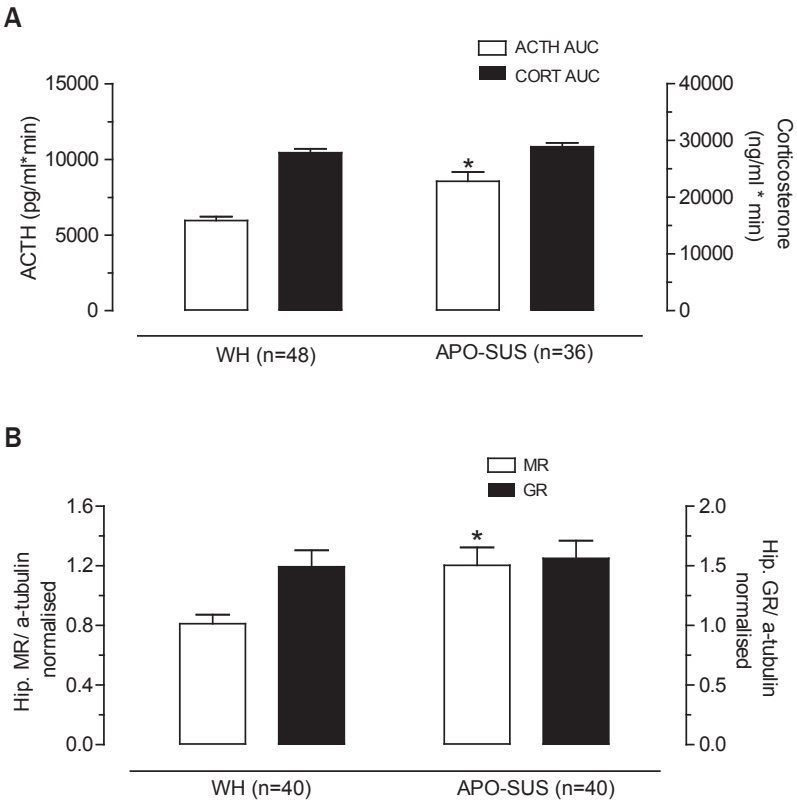

Figure 2. (A) Endocrine response to contextual fear (ACTH and Corticosterone area under the curve) and (B) basal hippocampal MR and GR protein levels of apomorphine susceptible rats (APO-SUS) and common Wistar Hannover rats (WH). Data presented as MEAN ± SEM. * vs. corresponding values of WH. The exact number of rats used is indicated in the different panels.

3.1.3 Acute effects of CORT on APO-gnawing

When the APO-gnawing measurement was repeated, the gnawing counts stayed the same for both rat genotypes (Fig. 3A). In both times of testing (1st & 2nd), there was a significant rat genotype effect ($F_{1,83}$= 697.960; p<0.001/ p<0.001 both times). However, if, one hour before the repetition of the APO-gnawing test, rats were pretreated with a high concentration of CORT, there was a difference in the gnawing counts (Fig. 3B). Two-way ANOVA analysis revealed effects of both CORT injection ($F_{1,249}$=3.890; p<0.001) and rat genotype ($F_{1,249}$=1303.944; p<0.001), but not of their interaction. The CORT effect was significant only for the control WH rats (p=0.001) that increased their gnaw counts after CORT pretreatment. Rat genotype effect was significant at both time points (p<0.001).

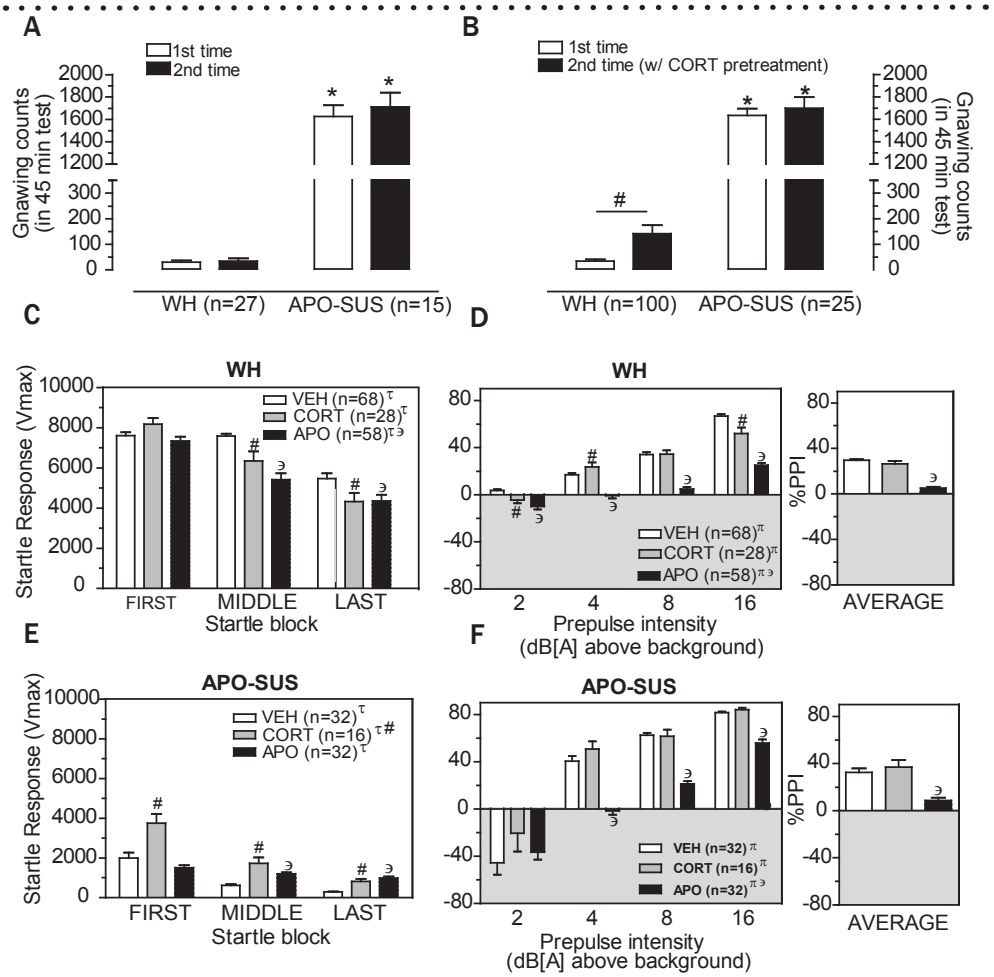

Figure 3. Acute effects of CORT. Effect of repetition and CORT pre-treatment on apomorphine-induced gnawing (A,B) of apomorphine susceptible Wistar rats (APO-SUS) and common Wistar rats (WH); Acoustic startle (C,E), and prepulse inhibition (D,F) of apomorphine susceptible rats (APO-SUS) and common Wistar Hannover rats (WH) injected with vehicle (VEH), Corticosterone 3 mg/kg (CORT) or Apomorphine 0.5 mg/kg (APO). Left panel D, F show PPI expressed per prepulse intensity level, whereas right panel D, F show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. * vs. corresponding values of WH, # CORT vs. 1st time or CORT vs. corresponding values of VEH, ə APO vs. corresponding values of VEH, τ denotes startle block effect, π denotes prepulse intensity effect. The exact number of rats used is indicated in the different panels.

3.1.4 Acute effects of CORT on sensorimotor gating

3.1.4.1 WH Acoustic Startle (Fig. 3C). All the injected groups displayed a significant effect of startle block, indicating habituation ($p<0.001$). There was an interaction effect of CORT injection with the startle block effect ($F_{2,188}=10.21$; $p<0.001$). The CORT injected WH rats startled less than the VEH injected in the middle ($p=0.001$) and last startle block

(p=0.028). There was a significant effect of APO injection ($F_{1,125}$=19.81; p<0.001) and its interaction with time ($F_{2,248}$=14.31; p<0.001). The APO injected WH rats startled less than the VEH injected in the middle (p<0.001) and last startle block (p=0.008).

3.1.4.2 WH PPI (Fig. 3D). All the injected groups displayed a significant effect of prepulse intensity (p<0.001). There was an interaction effect of CORT injection with the prepulse intensity effect ($F_{3,282}$=10.67; p<0.001). CORT injected WH displayed lower PPI than the VEH injected in low and high prepulse intensities (2: p=0.002, 16: p<0.001), but higher in medium (4: p=0.047). Therefore the overall PPI average of the two groups was not different. There was a significant effect of APO injection on PPI ($F_{1,125}$=210.96; p<0.001) and its interaction with prepulse intensity ($F_{3,372}$=138.99; p<0.001). The APO injected rats displayed lower PPI than the VEH injected in all prepulse intensities apart from 8dB[A] (2: p=0.002 4: p=0.047, 16: p<0.001) and in average ($F_{1,125}$=296.10; p<0.001).

3.1.4.3 APO-SUS Acoustic Startle (Fig. 3E). All the injected groups displayed a significant effect of startle block, indicating habituation (p<0.001). There was a significant CORT effect in startle ($F_{1,47}$=28.84; p<0.001), which interacts with the startle block effect ($F_{2,92}$=4.15; p=0.019). The CORT injected APO-SUS rats startled more than the VEH injected in all the startle blocks (first-middle-last: p≤0.001). There was a significant interaction effect of APO injection and time ($F_{2,124}$=17.23; p<0.001). The APO injected APO-SUS rats startled more than the VEH injected in the middle and last startle block (p<0.001).

3.1.4.4 APO-SUS PPI (Fig. 3F). All the injected groups displayed a significant effect of prepulse intensity (p<0.001). There was no significant effect of CORT injection in APO-SUS PPI. However, there is a significant effect of APO injection ($F_{1,63}$=30.87; p<0.001) and its interaction with time ($F_{3,186}$=15.66; p<0.001). The APO injected rats displayed lower PPI than the VEH injected in all prepulse intensities apart from 2dB[A] (4-8-16: p<0.001) and in average ($F_{1,63}$=33.54; p<0.001).

3.2 Experiment II - Genotype-dependent differences in maternal care

3.2.1 Maternal Behavior Average (Fig. 4A-E)

One-way ANOVA revealed effect of rat genotype in the average of all maternal behaviors the first week after parturition (AN: $F_{1,49}$=25.16; p<0.001, PN: $F_{1,49}$=27.89; p<0.001, Away: $F_{1,49}$=11.97; p=0.001, LG: $F_{1,49}$=7.61; p=0.008, SG: $F_{1,49}$=27.74; p<0.001). APO-SUS dams were more times away from their nest or engaged in self-grooming than the WH dams, while they spent less time in nursing (active or passive) and LG.

3.2.2 Maternal Behavior Variation (Fig. 4F-J)

One-way ANOVA revealed effect of rat genotype in the variation of PN ($F_{1,49}$=21.62; p<0.001), Away ($F_{1,49}$= 5.88; p=0.019), and SG ($F_{1,49}$=41.86; p<0.001). APO-SUS displayed reduced variation of PN, and increased variation of time away and self-grooming compared to WH.

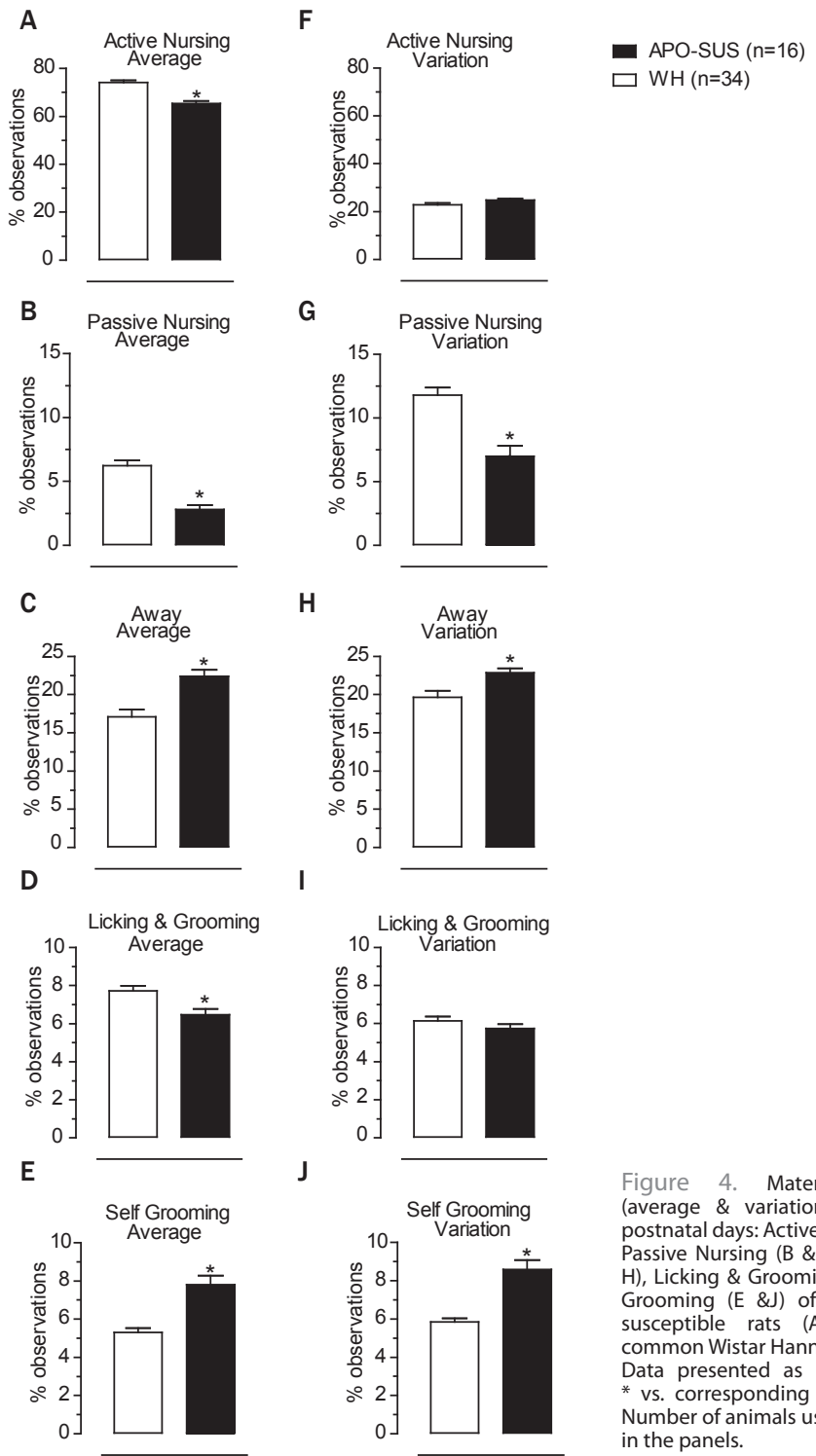

Figure 4. Maternal behavior (average & variation) the first 7 postnatal days: Active Nursing (A&F), Passive Nursing (B & G), Away (C & H), Licking & Grooming (D & I), Self Grooming (E &J) of apomorphine susceptible rats (APO-SUS) and common Wistar Hannover rats (WH). Data presented as MEAN ± SEM. * vs. corresponding values of WH. Number of animals used is indicated in the panels.

3.2.3 Maternal Behavior Time Course

AN (Fig. S5A): Repeated measures one way ANOVA revealed effect of time ($F_{34,1632}$=27.43; p<0.001), rat genotype ($F_{1,49}$= 5.92; p=0.019), and their interaction ($F_{34,1632}$=4.10; p<0.001). The time effect was significant for both rat genotypes (p<0.001) and further analysis revealed rat genotype effects in many individual time points (Fig. S5A), where the APO-SUS dams displayed less AN than the WH.

PN (Fig. S5B): Repeated measures one way ANOVA effect of time ($F_{34,1632}$=2.70; p<0.001), rat genotype ($F_{1,49}$= 27.90; p<0.001) and their interaction ($F_{34,1632}$=1.56; p=0.021). The time effect was significant for both rat genotypes (p<0.001) and further analysis revealed rat genotype effects in many individual time points (Fig. S5B), where the APO-SUS dams displayed less PN than the WH.

Away (Fig. S5C): Repeated measures one way ANOVA revealed effect of time ($F_{34,1632}$=37.31; p<0.001), rat genotype ($F_{1,49}$= 11.97; p=0.001), and their interaction ($F_{34,1632}$=5.34; p<0.001). The time effect was significant for both rat genotypes (p<0.001) and further analysis revealed rat genotype effects in many individual time points (Fig. S5C), where the APO-SUS dams were more often away from the nest than the WH.

LG (Fig. S5D): Repeated measures one way ANOVA revealed effect of time (F34,1632=2.18; p<0.001), rat genotype ($F_{1,48}$= 7.60; p=0.008) and their interaction ($F_{34,1632}$=1.76; p=0.005). The time effect was significant for both rat genotypes (p<0.001) and the further comparisons and further analysis revealed rat genotype effects in many individual time points (Fig. S5D), where the APO-SUS dams displayed less LG than the WH.

SG (Fig. S5E): Repeated measures one way ANOVA revealed effect of time ($F_{34,1632}$=8.49; p<0.001), and their interaction ($F_{34,z632}$=3.70; p<0.001). The time effect was significant for both rat genotypes (p<0.001) and further analysis revealed rat genotype effects in many individual time points (Fig. S5E), where the APO-SUS dams displayed more SG than the WH.

3.3 Experiment III - interaction of genetic susceptibility with early-life stress (Hit 1 & 2)

3.3.1 Developmental parameters

3.3.1.1 Body weight (Table S2). From pnd 11, Low LG offspring were significantly lighter than High LGs and this persists. We have followed and confirmed this maternal care history induced difference in body weight, on a weekly basis, up to pnd 165.

3.3.1.2Neonatal response to novelty-stress (pnd 5). ACTH (Fig. S3E): Two-way ANOVA revealed an effect of maternal care history ($F_{1,31}$=4.45; p=0.044) and novelty-stress ($F_{1,31}$=11.40; p=0.002), but not of their interaction. Both LG groups responded with an increase of ACTH to 30min of novelty (High LG: p=0.026, Low LG: p=0.038).

CORT (Fig. S3F): Two-way ANOVA revealed an effect of novelty-stress ($F_{1,31}$=13.21;

p=0.001), but not of maternal care history or their interaction. Both LG groups responded with an increase of CORT to 30min of novelty (High LG: p=0.017, Low LG: p=0.028).

3.3.1.3 TH protein levels (Fig. S3F). One-way ANOVA did not reveal an effect of maternal care history.

3.3.1.4 Eye-opening (Fig. S3H). One-way ANOVA did not reveal an effect of maternal care history.

3.3.2 Behavior/ Endocrine measurements

3.3.2.1 Basal APO-gnawing (data not shown). Low LG offspring displayed less gnaw counts than the High LG ($F_{1,35}$=11.10; p=0.002). However, both groups displayed the extremely high gnawing levels typical for APO-SUS rats.

3.3.2.2 Basal Sensorimotor gating. Acoustic Startle (Fig. 5A): There was a significant effect of startle block within the protocol on acoustic startle for both LG groups reflecting habituation ($F_{2,68}$=106.95; p<0.001/ p<0.001 for both LG groups). The maternal care history had a significant effect, as well as its interaction with the startle block effect ($F_{1,35}$=13.01; p=0.001, $F_{2,68}$=30.18; p<0.001 respectively). Low LG offspring startled less

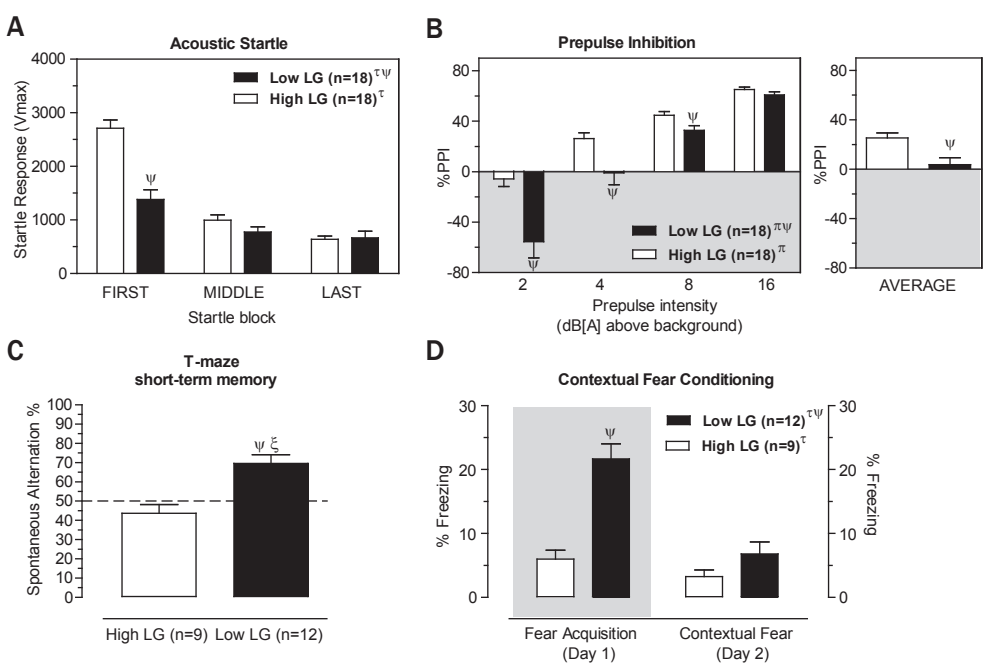

Figure 5. Behavior: Acoustic startle (A), Prepulse inhibition (B), T-maze short-term memory (C), 24h Contextual Freezing (D) of Low LG and High LG socially reared APO-SUS offspring. Left panel B show PPI expressed per prepulse intensity level, whereas right panel B show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. ψ vs. corresponding values of High LG, τ denotes startle block effect, π denotes prepulse intensity effect, ξ vs. 50% (chance level). The exact number of rats used is indicated in the different panels.

than the High LG offspring, but only in the first startle block ($p<0.001$).

PPI (Fig. 5B): There was a significant effect of the increasing prepulse intensity on PPI in both LG groups ($F_{3,102}=112.350$; $p<0.001$/ $p<0.001$ for both LG groups). The maternal care history and its interaction with the prepulse intensity effect had also significant effects ($F_{1,35}=13.39$; $p=0.001$, $F_{3,102}=6.73$; $p<0.001$ respectively). Low LG offspring displayed lower PPI than the High LG offspring in all pre-pulse intensities apart from 16dB[A] (2:$p=0.001$, 4: $p=0.013$, 8: $p=0.017$) and overall ($F_{1,35}=9.85$; $p=0.003$).

3.3.2.3 T-maze short-term memory (Fig. 5C). Low LG offspring displayed more spontaneous alternation than the High LG offspring ($F_{1,20}=8.67$; $p=0.008$) and higher than the 50% chance level ($p<0.001$). High LG offspring performed at the 50% chance level.

3.3.2.4 Conditioned emotional response. Behavioral (Fig. 5D): Repeated measures one-way ANOVA revealed a main effect of time ($F_{2,38}=22.62$; $p<0.001$/ Low LG: $p<0.001$, High LG: $p=0.005$), maternal care history ($F_{1,20}=4.82$; $p=0.041$), and their interaction ($F_{2,38}=9.26$; $p=0.001$). For the post-shock 2min period, Low LG offspring froze more than the High LG ($p<0.001$), indicating differences in the acquisition of fear. For the first 2min and the last 2min of re-exposure (data not shown), there was not a difference in freezing between the two LG groups.

Endocrine. ACTH response (Fig. 6A): Repeated measures one-way ANOVA revealed a main effect of time ($F_{4,76}=29.90$; $p<0.001$/ $p<0.001$ for both LG groups), maternal care history ($F_{1,20}=39.76$; $p<0.001$), and their interaction ($F_{4,76}=15.84$; $p<0.001$). Except of 4min after stress time point, Low LG offspring displayed higher ACTH levels than High LG offspring (baseline: $p=0.001$, 10min $p=0.002$, 15min: $p<0.001$ & 30min: $p=0.001$). ACTH AUC (Fig. 6D): Low LG offspring displayed greater total ACTH output than the High LG offspring ($F_{1,20}=41.74$; $p<0.001$).

Prolactin release (Fig. 6B): Repeated measures one-way ANOVA revealed a main effect of time ($F_{4,76}=13.04$; $p=0.015$/ High LG: $p<0.001$, Low LG: $p=0.039$), maternal care history ($F_{1,20}=6.12$; $p=0.023$) and their interaction ($F_{4,76}=13.04$; $p=0.002$). Low LG offspring displayed lower prolactin levels than High LG offspring at basal conditions ($p=0.020$) and higher at 30min after stress ($p=0.001$).

CORT response (Fig. 6C): Repeated measures one-way ANOVA revealed a main effect of time ($F_{5,95}=148.26$; $p<0.001$/ $p<0.001$ for both LG groups), maternal care history ($F_{1,20}=16.11$; $p=0.001$), and their interaction ($F_{5,95}=11.64$; $p<0.001$). Apart from baseline and 120min after stress, Low LG offspring displayed higher CORT levels than the High LG offspring (4min: $p=0.007$, 10min: $p=0.046$, 15min: $p=0.001$, 30min: $p<0.001$). At 120 min after stress time point they actually displayed lower levels than High LG offspring ($p<0.001$). CORT AUC (Fig. 6D): Low LG offspring displayed higher CORT output than the High LG offspring ($F_{1,20}=15.23$; $p=0.001$).

MR and GR protein levels. Hippocampus MR and GR levels (Fig. 6E): One-way ANOVA did not reveal any effect of maternal care history on hippocampal levels of MR, GR or their ratio (data not shown). Pituitary GR levels (data not shown): There was no

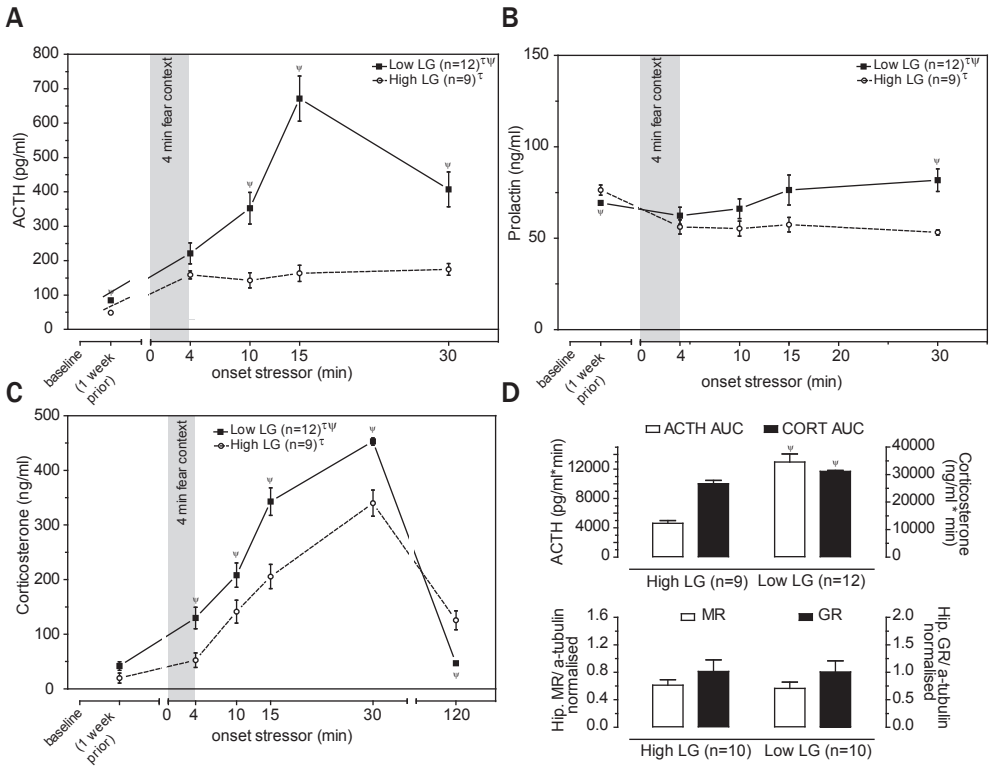

Figure 6. Endocrine response to contextual fear: ACTH (A), Prolactin (B), Corticosterone (C), ACTH and Corticosterone area under the curve (D), basal MR and GR protein levels (E) of Low LG and High LG socially reared APO-SUS offspring. Data presented as MEAN ± SEM. ψ vs. corresponding values of High LG, τ denotes time effect. The exact number of rats used is indicated in the different panels.

difference in GR levels between the two genotypes.

3.3.3 Acute effects of CORT on sensorimotor gating

3.3.3.1 Low LG offspring Acoustic Startle (Fig. 7A). All the injected groups displayed a significant effect of startle block, indicating habituation (VEH: p<0.001, CORT: p=0.001, APO: p=0.002). There was a significant CORT effect in startle ($F_{1,26}$=25.16; p<0.001), which interacts with the startle block effect ($F_{2,50}$=7.76; p=0.001). The CORT injected Low LG offspring startled more than the VEH injected in all the startle blocks (first-middle-last: p=0.001). The APO injected Low LG offspring startled more than the VEH injected in the last startle block (p<0.001).

3.3.3.2 Low LG offspring PPI (Fig. 7B). All the injected groups displayed a significant effect of prepulse intensity (p<0.001). The CORT injected Low LG offspring displayed lower PPI than the VEH injected only in 8dB[A] prepulse intensity (p=0.045). There was a significant effect of APO injection ($F_{1,26}$=59.75; p<0.001). The APO injected Low LG offspring displayed lower PPI than the VEH injected in all prepulse intensities (2: p=0.021, 4-8-16: p<0.001) and in average ($F_{1,26}$=52.93; p<0.001).

3.3.3.3 High LG offspring Acoustic Startle (Fig. 7C). All the injected groups displayed a significant effect of time, indicating habituation ($p<0.001$). There was a significant CORT injection effect in startle ($F_{1,26}=23.41$; $p<0.001$), which interacts with the time ($F_{2,50}=20.32$; $p<0.001$). The CORT injected High LG offspring startled more than the VEH injected in the first and last startle blocks (first: $p=0.001$, last: $p=0.002$). There was a significant interaction effect of APO injection and time on startled ($F_{2,50}=18.68$; $p<0.001$). The APO injected High LG offspring startled more than the VEH injected in the first startle block ($p=0.002$) and startled less in the last startle block ($p=0.001$)

3.3.3.4 High LG offspring PPI (Fig. 7D). All the injected groups displayed a significant effect of prepulse intensity ($p<0.001$). There was a significant effect of CORT injection ($F_{1,26}=6.36$; $p=0.018$). The CORT injected High LG offspring displayed lower PPI than the VEH injected in all prepulse intensities apart from 4dB[A] prepulse intensity (2: $p=0.029$, 8: 0.017, 16: $p<0.001$) and in overall average ($F_{1,26}=6.37$; $p=0.018$). There was a significant effect of APO injection ($F_{1,26}=51.05$; $p<0.001$). The APO injected High LG offspring displayed lower PPI than the VEH injected in all prepulse intensities (2:

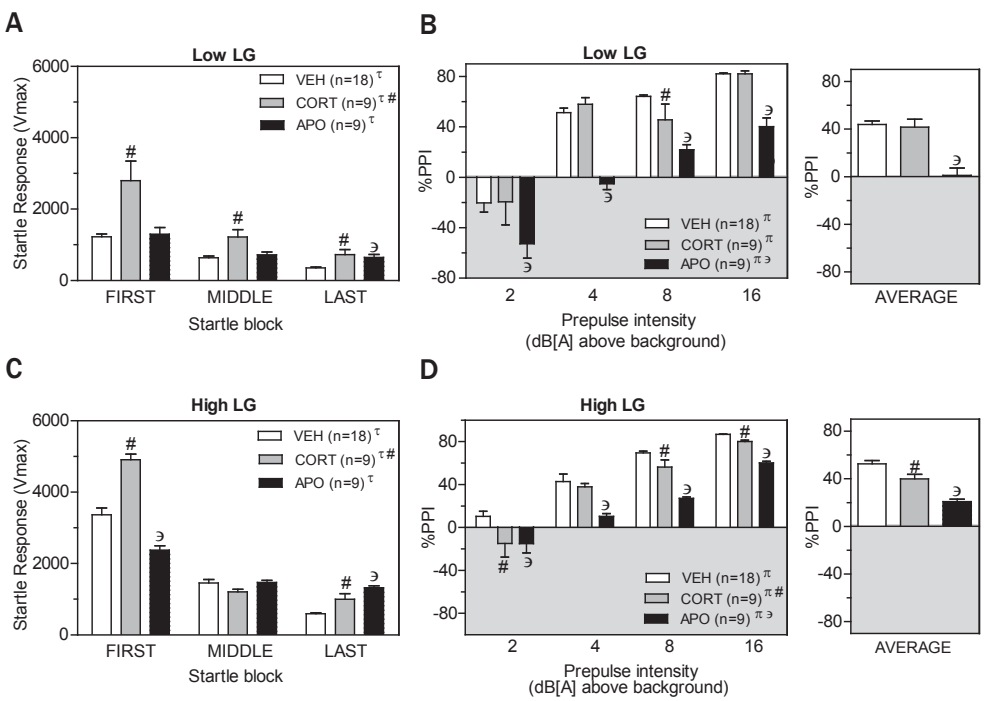

Figure 7. Acoustic startle (A,C), and Prepulse inhibition (B,D)) of Low LG and High LG socially reared APO-SUS offspring injected with vehicle (VEH), Corticosterone 3 mg/kg (CORT) or Apomorphine 0.5 mg/kg (APO). Left panel B, D show PPI expressed per prepulse intensity level, whereas right panel B, D show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. # CORT vs. corresponding values of VEH, ə APO vs. corresponding values of VEH, τ denotes startle block effect, π denotes prepulse intensity effect. The exact number of rats used is indicated in the different panels.

p=0.008, 4: p=0.004, 8-16: p<0.001) and in average ($F_{1,26}$=51.05; p<0.001).

3.4 Experiment IV - Interaction of genetic susceptibility and post-weaning social environment (Hit 1 & 3)

3.4.1 Body weight (Table S3)

Med LG isolates were heavier than Med LG socials in the peri-pubertal period.

3.4.2 Basal sensorimotor gating

3.4.3.1 Acoustic Startle (Fig. S6A). There was a significant effect of startle block on startle within the PPI protocol for all Med LG groups reflecting habituation (socials-breeders-isolates p<0.001, adult isolates p<0.004). The breeders startled more than the socials in the last startle block of the protocol (p=0.028). Isolation in adulthood had a significant effect in startle ($F_{2,31}$=5.57; p=0.025). Adult isolates startled more than socials in the middle (p=0.008) and last startle block (p=0.021) of the protocol. Isolation rearing had a significant effect in startle ($F_{2,37}$=7.56; p=0.009). Isolates startled less than the socials in the middle startle block of the protocol (p<0.001). Finally, isolation rearing compared to isolation in adulthood had a significant effect in startle ($F_{2,33}$=15.34; p<0.001) which interacted with the time effect ($F_{2,64}$=3.16; p=0.049). Isolates startled less than adult isolates in all startle blocks of the protocol (first: p=0.007, middle: p<0.001, last: p=0.003).

2.4.3.2 PPI (Fig. S6B). There was a significant effect of increasing prepulse intensity on PPI in all Med LG groups (p<0.001 for all the groups). Breeders were not different from socials in any PPI measure. Isolation in adulthood had an effect on PPI that interacted with the prepulse effect ($F_{3,90}$=4.49; p=0.006). Adult isolates displayed higher PPI in the higher prepulse intensities tested (8: p=0.023, 16: p=0.033). Isolation rearing had a significant effect ($F_{1,37}$=10.62; p<0.001), which interacted with the time effect ($F_{3,108}$=6.88; p<0.001). Isolates displayed lower PPI in the lower prepulse intensities tested (2dB[A]:p=0.002, 4dB[A]:p=0.002) and in overall average ($F_{1,37}$=32.04; p<0.001). Finally, isolation rearing compared to isolation in adulthood had a significant effect in PPI ($F_{1,33}$=9.38; p=0.004). Isolates displayed lower PPI than adult isolates in all the intensities tested apart from 4dB[A] (2:p=0.019, 8:p=0.004, 16:p=0.006) and in overall average ($F_{1,33}$=13.22; p=0.001).

3.4.3 Basal APO-gnawing (Fig. S6C)

Med LG isolates displayed the same gnaw counts with the Med LG socials (both in the high gnawing levels range typical for APO-SUS rats).

3.4.4 T-maze short-term memory (Fig. S6D)

Med LG isolates performed in a stereotyped fashion during the spontaneous alternation task (approx. 30% & significantly lower than 50% chance level; p=0.027). Their performance was significantly lower than the Med LG socials ($F_{1,35}$=42.05; p=0.001)

that performed higher than the 50% chance level (p=0.034).

3.5 Experiment V – Interaction of genetic susceptibility with both early-life stress and post-weaning social environment (Hit 1, 2 & 3):

3.5.1 Body weight (Table S4)

Low LG offspring (that were lighter than High LG offspring on pnd 11 & 21) raised post-weaning as isolates were not lighter than High LG isolates until the peri-pubertal period and early adulthood. After pnd 116 they were lighter than the High LG counterparts.

3.5.2 Basal sensorimotor gating

3.5.3.1 Acoustic Startle (Fig. 8A). There was a significant effect of startle block within the protocol on acoustic startle for both isolate LG groups reflecting habituation ($F_{2,32}$=46.49; p<0.001/ p<0.001 for both isolate LG groups). The maternal care history had also a significant effect ($F_{1,17}$=11.10; p=0.004). Low LG isolated offspring startled

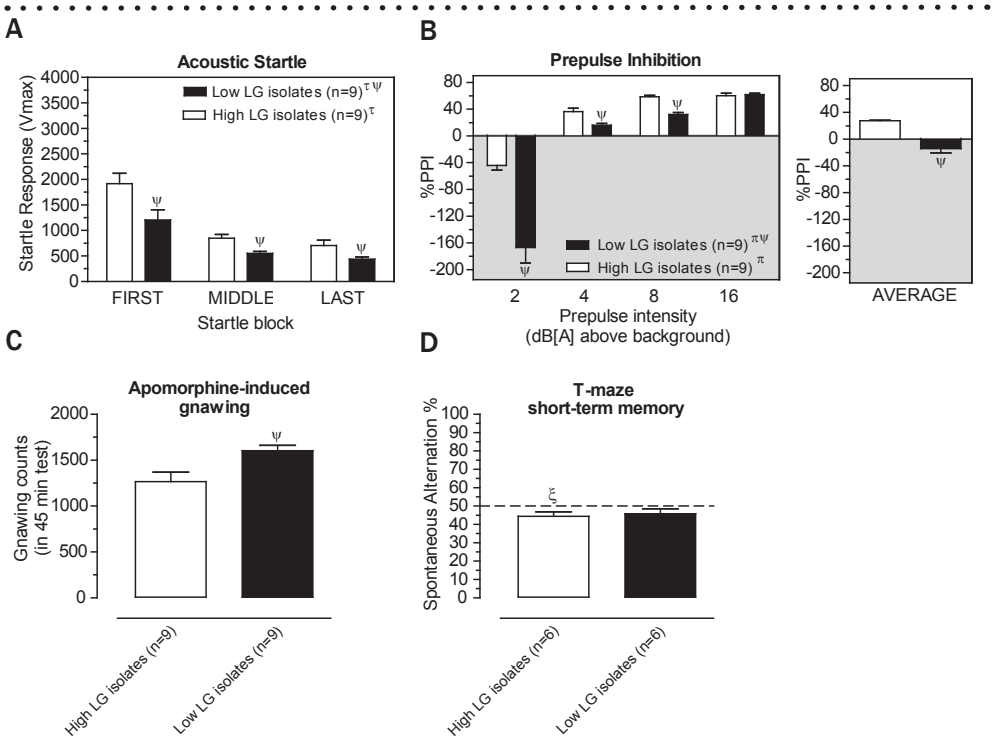

Figure 8. Behavior: Acoustic startle (A), Prepulse inhibition (B), Apomorphine induced gnawing (C), T-maze short-term memory (D) of Low LG and High LG APO-SUS isolates. Left panel B show PPI expressed per prepulse intensity level, whereas right panel B show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. ψ vs. corresponding values of High LG, τ denotes startle block effect, π denotes prepulse intensity effect, ξ vs. 50% (chance level). The exact number of rats used is indicated in the different panels.

less than the High LG isolates in all startle blocks (first: p=0.022, middle: p=0.002, last: p=0.027).

3.5.3.2 PPI (Fig. 8B). There was a significant effect of the increasing prepulse intensity on PPI in both isolated LG groups ($F_{3,48}$=146.08; p<0.001/ p<0.001 for both LG isolated groups). The maternal care history and its interaction with time had also a significant effect on PPI ($F_{1,17}$=42.05; p<0.001, $F_{3,48}$=19.17; p<0.001 respectively). Low LG isolates displayed lower PPI than the High LG isolates in all pre-pulse intensities apart from 16dB[A] over background (2: p<0.001 , 4: p=0.003 , 8: p<0.001) and in overall average ($F_{1,17}$=42.05; p<0.001).

3.5.3 Basal APO-gnawing (Fig. 8C)

Low LG isolates displayed higher gnaw counts than the High LG isolates ($F_{1,17}$=7.92; p=0.012). All groups displayed gnawing levels in the high gnawing level range typical for APO-SUS rats.

3.5.4 T-maze short-term memory (Fig.8D)

Low LG isolates were not significantly different from the High LG isolates in spontaneous alternation as ($F_{1,20}$=8.67; p=0.008). However, Low LG offspring performed at the chance level, whereas High LG offspring performed lower than the 50% chance level (p=0.049).

4. Discussion

The present study demonstrates schizophrenia-like vulnerability in genetically susceptible rats if they had experienced less maternal care and were subjected to post-weaning isolation rearing. The selected genetic predisposition is linked to hyper-responsiveness of the dopaminergic system to APO. This hyper-dopaminergic trait had as additional signature glucocorticoid resistance and adrenal hypo-responsiveness in the face of an exceptionally high stress-induced ACTH release, when combined with environmental stress in development. Taken together, these findings suggest that HPA-axis activity is implicated in individual differences in schizophrenia susceptibility and provides strong support for the *three-hit* hypothesis of psychopathology. Below we will discuss each 'hit' separately and conclude with a synthesis.

4.1 Hit 1: Genetic predisposition for APO-susceptibility

We characterized the phenotype of APO-SUS rats in comparison with rats from their paternal outbred Wistar population in order to extend previous comparisons with their APO-UNSUS counterparts to a commonly used outbred rat strain [16]. Body weight of the APO-SUS rats was remarkably reduced throughout life, which is not surprising given the established role of brain dopamine and DRD_2 in energy homeostasis [38, 39]. DRD_2 agonism decreases and antagonism increases body weight in humans and

rodents; polymorphisms of DRD_2 that reduce the DRD_2 function were associated with obesity and type 2 diabetes [39-41]. As expected the APO-SUS rats displayed very high APO-gnawing behavior, which was very low in the WH as previously observed in the APO-UNSUS. Previously, APO-SUS, compared to APO-UNSUS rats, displayed reduced freezing in response to social threat, an enhanced ACTH but blunted prolactin response to a conditioned emotional stressor [15, 18, 42]. We demonstrate in the present study that APO-SUS rats also display these characteristics in comparison with an outbred WH population.

However, we did not observe a basal PPI deficit of the APO-SUS rats compared to WH in contrast with a deficit found previously in comparison with the APO-UNSUS [17]. APO-SUS actually displayed the same or even higher levels of inhibition than the WH, while their acoustic startle response was low. Laboratory setting of the PPI measurement involving stressful influences could have influenced the latter result. Also the recent life history of the rat is an important determinant of the sensorimotor gating performance according to previous studies and our data in Med LG APO-SUS offspring. APO-SUS rats in the past were tested after one to three days of social isolation [17] and it is known that social isolation in adulthood of APO-SUS rats also can have a negative effect on sensorimotor gating [43]. Moreover, it cannot be ruled out that the APO-UNSUS might have been actually displaying enhanced PPI. This, however, needs further investigation with the use of all three genotypes (WH, APO-SUS, APO-UNSUS) in the same experiment. Finally, it cannot be excluded that subtle differences in breeding conditions and upbringing conditions (with possible epigenetic influence) did contribute to the observed absence of PPI disruption in the APO-SUS line.

Interestingly, the neonatal endocrine response, the adrenal TH levels and developmental somatic markers already demonstrated the APO-SUS phenotype. Compared to WH, the APO-SUS' ACTH response to novelty at pnd 5 was enhanced, adrenal TH protein levels were increased, the pups body weight was reduced starting from pnd 11 and their eye-opening was delayed one day (as was noted previously in [44, 45]). Hence, in line with the findings of Rots and colleagues (1995), it seems that peripheral endocrine changes in the APO-SUS appear very early and possibly precede the divergence in central dopamine responsiveness [46].

In conclusion, Hit 1: APO susceptibility appears not only to predispose for an enhanced response to APO, but also for glucocorticoid resistance, adrenal hypo-responsiveness, reduced fear and reduced acoustic startle. However, sensorimotor gating and short-term memory seem intact.

4.2 Hit 2: Maternal Care effects

The APO-SUS displayed lower levels of maternal care as expressed in LG, AN and PN, and spended more time away from the nest or in self-orientated behaviors. In previous studies maternal care of APO-SUS animals was not measured systematically, although it has been noted very early that APO-SUS mothers paid less attention to their pups

than the APO-UNSUS mothers [15] and displayed less time in blanket nursing and pup retrieval (Cools unpublished data). Cross-fostering of APO-SUS pups with APO-UNSUS dams affected later life outcome by attenuating the magnitude of APO-gnawing. Maternal deprivation had the opposite effect (increased APO-gnawing) in APO-UNSUS offspring, but no effect in APO-SUS offspring [19]. Possibly these effects in APO-SUS offspring could be explained by their low overall perceived maternal care by the APO-SUS dams.

To disentangle the effects of maternal care from that of genotype and genotype dependent reduction of maternal care we decided to study the effects of maternal care within the APO-SUS rat population, since they were bred to be genetically homogeneous. Pnd 1-7 LG was proven before, mainly in Long Evans rats, to be a sensitive period to study the effect of the quantity of maternal care within a certain rat population [26, 29]. Therefore, in our study Low LG APO-SUS offspring was compared to High LG offspring. The Low LG offspring displayed the lowest body weight for life, a much enhanced fear acquisition and a profoundly enhanced response of ACTH relative to CORT to a conditioned emotional stressor; they also showed basal PPI deficits, while their short-term spatial memory was enhanced. Even prolactin release, in response to stress, was enhanced in the Low LG APO-SUS offspring, apparently overriding the tonic inhibition of the hyperactive tuberinfundibular dopamine pathway of the APO-SUS rats [18, 47-49]. Taken together, the data show that the most severe phenotype within the APO-SUS individuals is linked to a condition of low maternal care in their neonatal life, reminiscent of previous findings with Long Evans rats [50, 51].

In conclusion Hit 2: We provide strong evidence that the APO-SUS individuals coming from litters of the low extreme of maternal care distribution display even more intense glucocorticoid resistance/adrenal hypo-responsiveness and even more reduced acoustic startle than the rest of the APO-SUS population. Interestingly, they also display the lowest levels of PPI, the best short-term spatial memory performance, and the highest fear acquisition within the APO-SUS population. An important strength of our study is that we replicated across two generations (F1 dams/F2 offspring & F2 dams/F3 offspring) the same maternal care effects.

4.3 Hit 3: Unfavorable social environment

Our model contains an interesting interaction between genetic and early-life adversity factors and we had the unique chance to test additionally if a later psychosocial stressor could amplify the observed behavioral deficits in the Low LG APO-SUS offspring. We first explored which social environment creates the greatest PPI deficit in APO-SUS rats irrespective of LG levels (Med LG rats were used). Isolation rearing disrupted clearly PPI of Med LG APO-SUS rats. Interestingly, isolation rearing also created a peri-pubertal increase in body weight and a reduction of T-maze spontaneous alternation (or even a stereotyped behavior in the T-maze – approx. 30% alternation) in adulthood. On the one hand, the isolation rearing effect on body weight, related to probably an increased

food intake, has been observed before and it coincides with a down-regulation of the hypothalamic CART-containing system [52]. On the other hand, the negative effect of isolation rearing on spatial memory is probably related to altered hippocampal and prefrontal functions [23, 24, 53].

We showed in agreement with previous studies that the isolation rearing has detrimental effects on PPI only when it starts from weaning (pre-pubertal period) [54, 55]. Further, Weiss and colleagues studied the effects of isolation rearing on PPI in different rat strains (Wistar, Sprague-Dawley and Lister hooded). Although they found a significant lowering effect of isolation on PPI in the Sprague-Dawley and Lister hooded rats, the isolated Wistar rats did not show a reduction in PPI [30, 56]. Interestingly, APO-SUS rats, coming from an original Wistar population, were sensitive to isolation rearing confirming the heightened sensitivity to environmental influence of this Wistar line [11, 57, 58].

We then used isolation rearing to modulate the behavioral deficits induced by Low LG in the APO-SUS offspring. We proved that, in genetically predisposed rats, two different stressful environmental experiences in development have additive effects. When we applied isolation rearing to Low and High LG APO-SUS offspring, we observed that the Low LG rats were very vulnerable to the effects of isolation rearing, while the High LG offspring were protected. Low LG APO-SUS offspring in isolation displayed a very severe phenotype that included: total absence of basal PPI, short-term memory impairment and an even further enhancement of the gnawing response to APO. Isolation rearing increased the body weight of the Low LG offspring in the peri-pubertal period and early adulthood. However, after this short time period, the Low LG offspring displayed again the lowest weight.

In conclusion, Hit 3: isolation rearing precipitates schizophrenia-like endophenotypes in APO-SUS individuals and even more in the Low LG offspring of the APO-SUS population with biomarkers spanning from DA-hypersensitivity to cognitive impairment.

4.4 Acute CORT effects on psychosis-susceptibility (induced by genotype +/- early-life adversity)

The APO-gnawing and PPI of the APO-SUS rats was not influenced by CORT injected 60min and 10min prior testing (respectively). This confirms and extends the CORT resistance of APO-SUS rats as noted previously [59]. A fast effect of CORT on PPI was, however, present in the control rats that were not sensitive to APO. This finding is reminiscent to a recent observation in healthy human volunteers with no previous drug abuse or family predisposition for psychosis, where likewise acute cortisol IV administration also disrupted PPI [60]. It would be interesting to investigate CORT acute effect in psychiatric populations (e.g. schizophrenia) or in individuals with personality traits linked to psychosis (e.g. schizotypy) or sensitivity to psychostimulants (e.g. sensation seeking, novelty seeking) [61, 62]. Taken together, acute CORT enhance

psychosis-susceptibility in animals with a relatively low inherited dopaminergic tone, which is in line with previous research by Piazza et al. using the high and low responders to amphetamine [63]. This CORT effect is fast and therefore possibly mediated by the recently identified membrane variant of the nuclear CORT receptors in brain (65, 66).

By injecting CORT in Low and High LG APO-SUS offspring we attempted to link sensorimotor gating deficits with the stress hormone in order to unravel a possible mechanism for psychosis pathogenesis [9]. The Low-LG APO-SUS offspring were CORT resistant judged from their stress-induced HPA-axis profile and High LG APO-SUS offspring were not. Hence, it is not surprising that their PPI phenotype did not further deteriorate with a CORT injection, while the one of High LG offspring did. Administration of antagonists for CORT receptors is expected to prevent either the basal PPI deficit in the Low LG offspring or the CORT induced disruption of the High LG offspring.

4.5 Synthesis: *Three-hit* hypothesis of schizophrenia

The *three-hit* or *cumulative stress* hypothesis states that, in genetically susceptible individuals, vulnerability for psychopathology precipitates after multiple events of environmental stress applied early or late in development [64, 65]. In our experiments, on the one hand, we observed an accumulation of detrimental effects of developmental stress in the APO-SUS genetic susceptibility. Early-life adversity (Low LG) enhances vulnerability of the genetically susceptible individuals (APO-SUS) to a later psycho-social stressor (isolation rearing) resulting in a severe schizophrenia-like phenotype. On the other hand, APO-SUS genetic susceptibility also heightened sensitivity to the positive effects of increased maternal care. High LG offspring individuals had increased basal PPI. They were protected from the detrimental effects of isolation rearing and their PPI was disrupted only after the administration of a high dose of the stress hormone.

5. Conclusion

A severe schizophrenia-like phenotype precipitates, if genetically predisposed individuals are cumulatively exposed to early adversity and a chronic psycho-social stressor initiated at juvenility. Genetically selected "reactive" dopaminergic alleles amplify the individual's vulnerability to schizophrenia–like phenotypes after cumulative exposure to stressors. The HPA-axis dysregulation and glucocorticoid resistance underscore the observed gene-by-environment interactions providing clues for the future on the biological basis of an "affective pathway for psychosis" [8].

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Supplementary Information

S1. Supplementary Methods & Materials

S.1.1 Naturally occurring maternal environment

F1 Cohort of APO-SUS females (n=16): Using the cumulative scores of pnd1-7, we were able to obtain 3 High LG litters (18.75%) with a mean LG of 8.14 ± 0.30 (% observations), 9 Medium LG (56.25%) with a mean LG of 6.74 ± 0.14 (% observations), and 4 Low LG litters (25.00%) with a mean LG of 4.68 ± 0.21 (% observations). The distribution of LG for this cohort is shown in Figure S1.The High LG and Low LG litters were used in Experiment III & V (see experimental design; Fig. S2). The Med LG litters were used in Experiment IV (see experimental design; Fig. S2).

F2 Cohort of APO-SUS females (n=20): In a subsequent cohort (20 APO-SUS litters), we followed the same procedure but for 4 days instead (pnd 1-4). We were able to obtain 5 High LG litters (25.00%) with a mean LG of 9.66 ± 0.49 (% observations), 11 Medium LG (55.00%) with a mean LG of 7.31 ± 0.21 (% observations), and 4 Low LG litters (20.00%) with a mean LG of 5.52 ± 0.34 (% observations). We double-checked the "pnd 1-4" LG limits with the values from the F1 cohort as described before [1]. On pnd 5, two Low LG litters with the lowest LG scores and two High LG litters with the highest LG scores were used in Experiment III (see experimental design).

From the remainder 16 litters, using the LG data from pnd 1-7, we were able to obtain 3 High LG litters (18.75%) with a mean LG of 9.19 ± 0.37 (% observations), 11 Medium LG (68.75%) with a mean LG of 6.95 ± 0.10 (% observations), and 2 Low LG litters (12.50%) with a mean LG of 4.70 ± 0.16 (% observations). The High LG and Low LG litters were used in Experiment III & V (see experimental design; Fig. S2). The Med LG litters were used in Experiment IV (see experimental design; Fig. S2).

Pearson correlations: Both cohorts showed significant Pearson correlations of cumulative LG scores of pnd 1-4 and pnd 1-7 (F1 cohort: $r=0.86$; $p\leq0.001$, F2 cohort: $r=0.80$; $p\leq0.001$) as described in literature before [2]. In cohort 2, the correlation was performed for 16 litters, because 4 litters as stated above were used in a test, which required the decapitation of the pups on pnd 5 and therefore maternal care measurements were stopped for these litters that day.

S.1.2 Tyrosine hydroxylase (TH), mineralocorticoid receptor (MR) & glucocortocoid receptor (GR) Western blotting

Adrenals and hippocampi were homogenized in 400 µl lysis buffer (Triethanolamine, NaCl, DOC, SDS, triton-X-100) and protease inhibitor was added to inhibit proteins' degradation. This lysate was spun down and supernatant was kept and stored in -20°C. Concentration of proteins present in the supernatant was determined using a Thermo Scientific Pierce BCA Protein Assay. Therefore, a calibration curve (Bovine Serum Albumin in 5 dilutions) was done.

The lysates were analysed by Western blotting, according to a previously described method [3], in duplicate. Each sample was loaded in a concentration of 1mg/ml. The samples also included a standard volume of sample buffer and were denaturized at 95°C (5min) and subjected to SDS–PAGE.

After electrophoresis, the proteins were transferred to a membrane (blotting) overnight (4°C,

125mA). The day after, the blots were blocked in 10mM Tris-HCl (pH 8.0), 150mM NaCl, and 0.05% Tween 20 containing 5% non-fat dried milk owder and, then, incubated with the primary antibody and the secondary antibody consecutively. For TH detection, the primary antibody used was a rabbit antibody (AB152) ordered from Millipore in a 1:1000 concentration. The secondary antibody used was Goat-anti-rabbit IgG-HRP in a 1:5000 concentration. We used mouse liver tissue as negative control and adult rat adrenal tissue as positive control. For MR detection, the primary antibody used was mouse monoclonal 1D5 1-18 [4] in a 1:1000 concentration. The secondary antibody used was goat anti mouse IgG-HRP in a 1:5000 concentration. We used untreated COS-cells as a negative control and COS-cells transfected with MR as a positive control. For GR detection, the primary antibody used was mouse monoclonal BuGR2 (ab2768) in a 1:1000 concentration, ordered from Abcam. The secondary antibody used was goat anti mouse IgG-HRP in a 1:5000 concentration. We used COS-cells with GR knockdown as a negative control and mouse liver tissue as a positive control. For loading control, samples were also tested on their α-tubulin levels. The primary antibody used was mouse in a 1:5000 concentration and the secondary antibody used was goat anti mouse IgG-HRP in a 1:10000 concentration.

After washing of the antibodies, blots were incubated with peroxidase-conjugated antibodies (1:10.000; Jackson ImmunoResearch Laboratories, West Grove, PA). The immunoreactive bands were visualized by enhanced chemiluminescence and the blots were exposed to films. The autoradiographs (films) were scanned and optical density (OD) of the TH, MR, GR and α-tubulin bands were determined using Image J software. The TH, MR and GR values of the samples were corrected for total protein (α-tubulin). In order to compare samples ran in different gels we used a sample that was loaded in all gels.

S2. Supplementary Figures

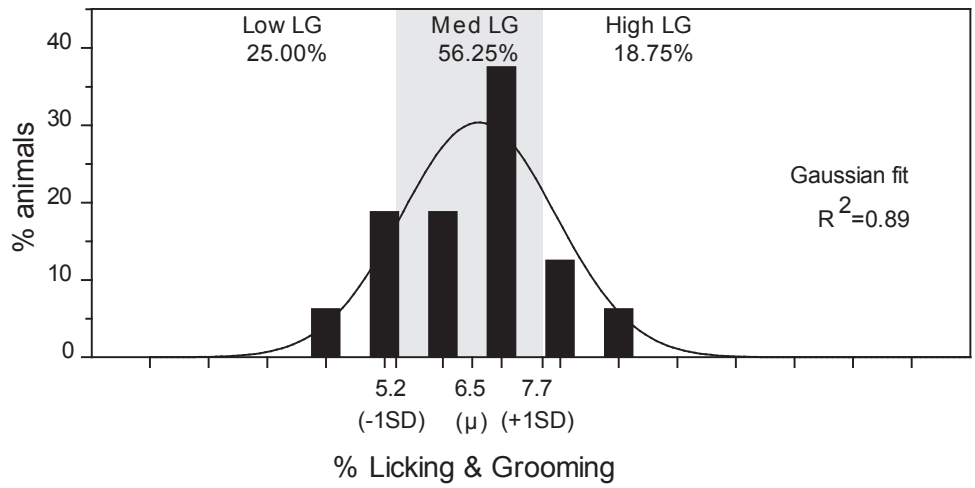

Figure S1. Frequency distribution of Licking & Grooming of apomorphine susceptible Wistar (APO-SUS) dams (n=16). On top of the distribution a normal distribution is superimposed with high Gaussian fit (R^2 =0.89). The mean of the population is indicated as well as the LG levels 1SD under and 1SD over the mean. These levels were used to divide the population in 3 groups: Low LG (25% of dams), Med LG (56.25% of dams) and High LG (18.75 % of dams).

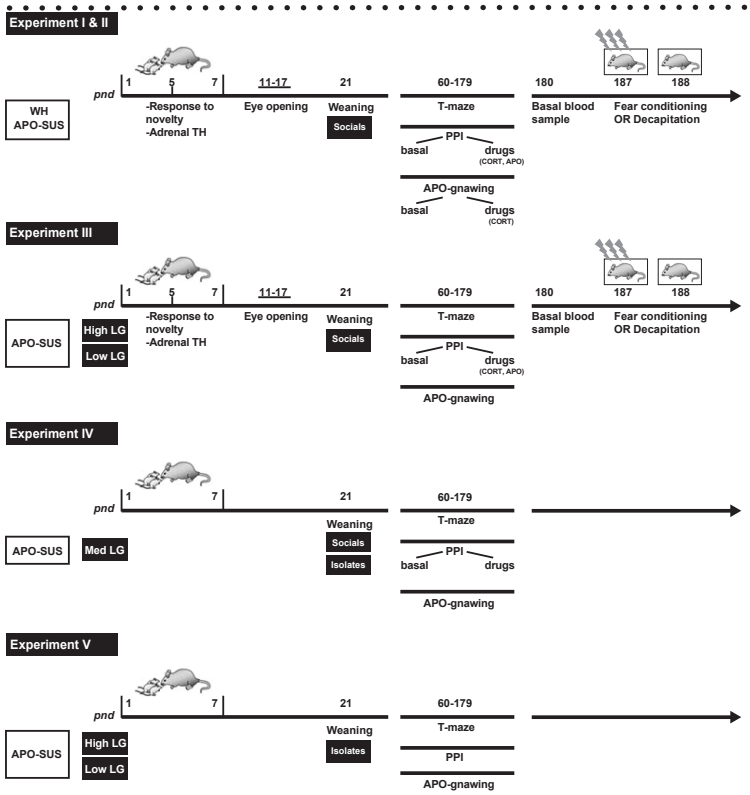

Figure S2. Graphical representation of the experiments. Experiment I & II: Time line of longitudinal study. Litters were divided to two treatment groups: Wistar Hannover (WH) and Apomorphine-Susceptible (APO-SUS) rats. Maternal care was observed at postnatal days (pnd) 1-7 (depicted with the design of the dam with the pups). On pnd 5 we measured the endocrine response of the pups to novelty and adrenal levels of TH. On pnd11-17, we observed the day of eye-opening. Weaning happened at pnd 21 and rats were housed in groups (socials). In the period of pnd 60-179 the behavioural testing was performed that included T-maze spontaneous alternation, PPI test (in basal conditions or after acute injection of drugs) and apomorphine-induced gnawing test (in basal or after CORT pretreatment). Basal blood sampling occurred at pnd 180 and fear conditioning or decapitation at pnd 187-188. Experiment III: Time line of longitudinal study. Maternal care was observed at postnatal days (pnd) 1-7. Litters were divided to two treatment groups: rats with history of High Licking & Grooming (High LG) and rats with history of Low LG (Low LG). On pnd 5 we measured the endocrine response of the pups to novelty and adrenal levels of TH. On pnd 11-17, we observed the day of eye-opening. Weaning happened at pnd 21 and rats were housed in groups (socials). In the period of pnd 60-179 the behavioural testing was performed that included T-maze spontaneous alternation, PPI test (in basal conditions or after acute injection of drugs) and apomorphine-induced gnawing test (in basal conditions). Basal blood sampling occurred at pnd 180 and fear conditioning or decapitation at pnd 187-188. Experiment IV: Time line of longitudinal study. Maternal care was observed at postnatal days (pnd) 1-7. Med LG rats were divided to two treatment groups according to the post-weaning (pnd 21) housing conditions: group housing (socials) and isolation rearing (isolates). In the period of pnd 60-179 the behavioural testing was performed that included T-maze spontaneous alternation, PPI test (in basal conditions or after acute injection of CORT) and apomorphine-induced gnawing test (in basal conditions). Experiment V: Time line of longitudinal study. Maternal care was observed at postnatal days (pnd) 1-7. Litters were divided to two treatment groups: rats with history of High Licking & Grooming (High LG) and rats with history of Low LG (Low LG). Weaning happened at pnd 21 and rats were housed individually (isolates). In the period of pnd 60-179 the behavioural testing was performed that included T-maze spontaneous alternation, PPI test (in basal conditions) and apomorphine-induced gnawing test (in basal conditions).

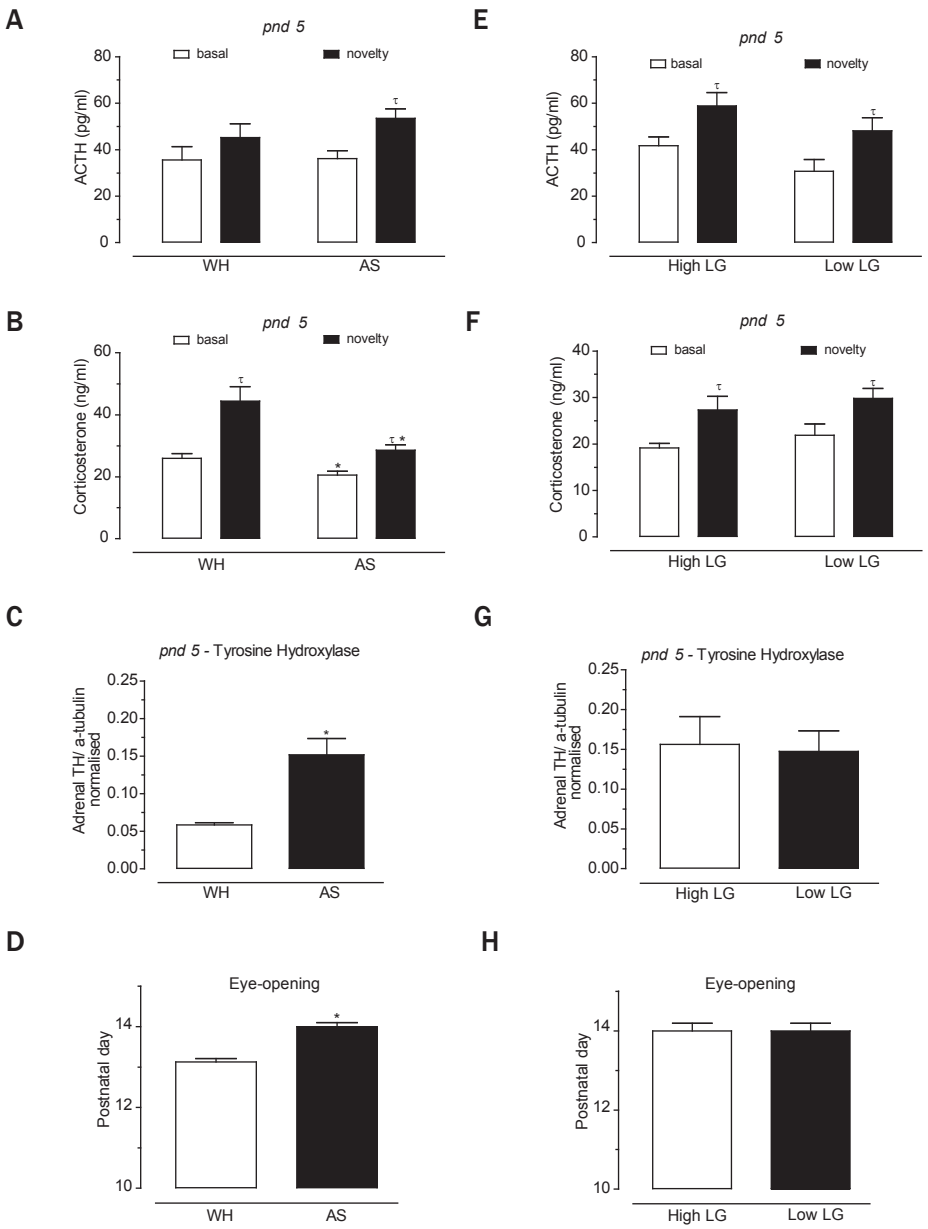

Figure S3. pnd 5 ACTH (A,E) & CORT (B,F) response to 30min novelty-stress, pnd 5 adrenal TH protein levels (C, G) and the pnd of eye opening (D, H) of common Wistar rats (WH) and apomorphine-susceptible Wistar rats (APO-SUS) or Low LG and High LG APO-SUS pups. Data presented as MEAN ± SEM. τ denotes novelty-stress effect, * vs. corresponding values of WH.

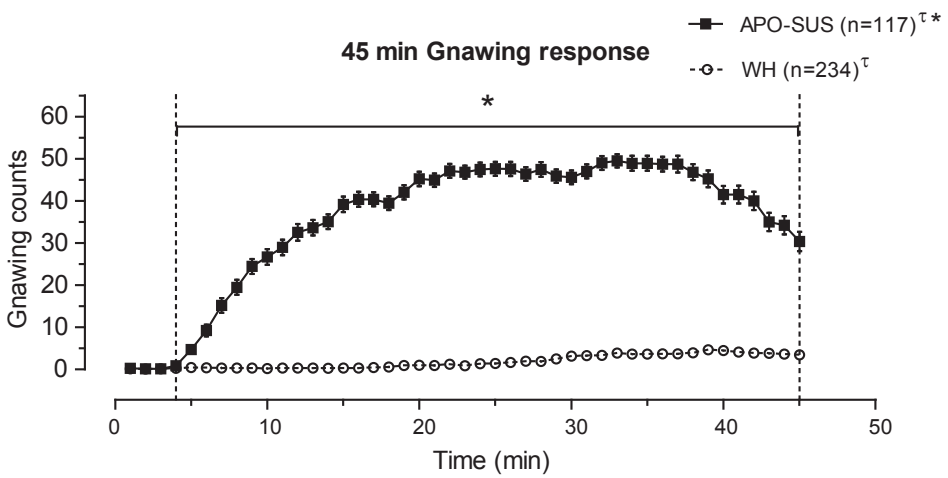

Figure S4. Apomorphine induced gnawing of apomorphine susceptible Wistar rats (APO-SUS) and common Wistar rats (WH). Gnawing counts within a 45 min observation period. Data presented as MEAN ± SEM. * vs. corresponding values of WH, τ denotes time effect. The exact number of rats used is indicated in the different panels.

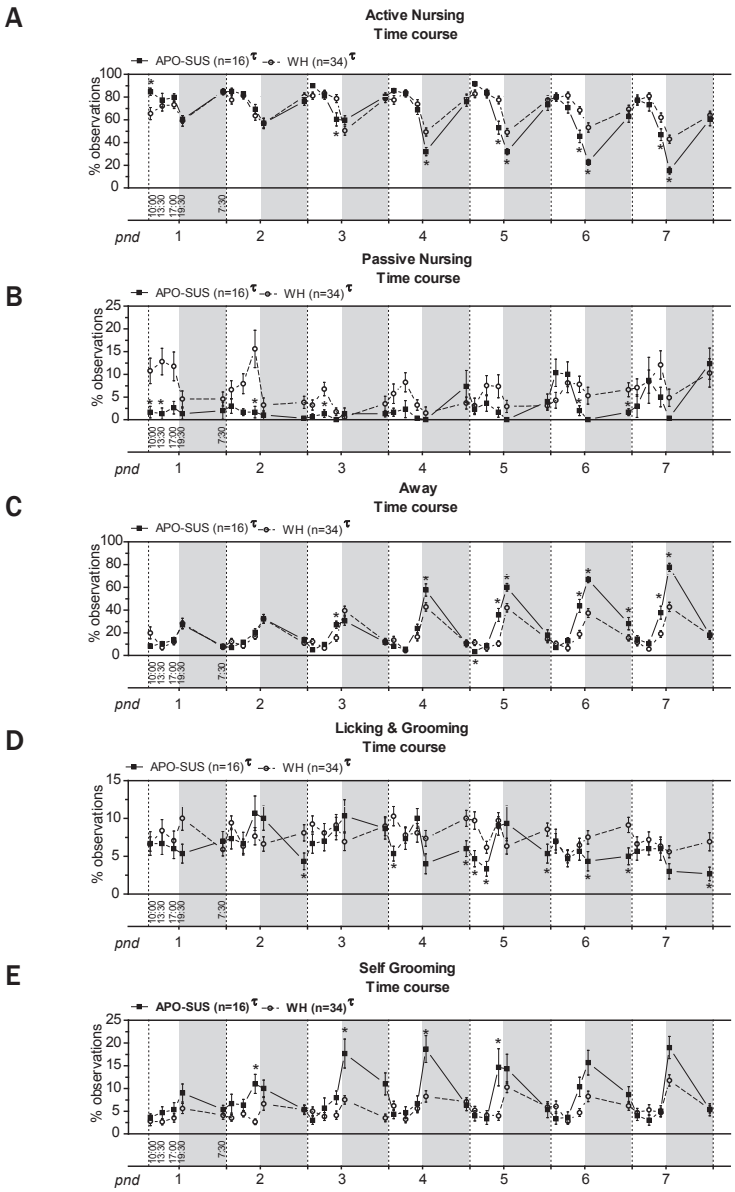

Figure S5. Maternal behavior time course the first 7 postnatal days (pnd): Active Nursing (A), Passive Nursing (B), Away (C), Licking & Grooming (D), Self Grooming (E) of apomorphine susceptible (APO-SUS) and common Wistar Hannover (WH) dams. Data presented as MEAN ± SEM. * vs. corresponding values of WH. τ denotes time effect. Number of animals used is indicated in the panels.

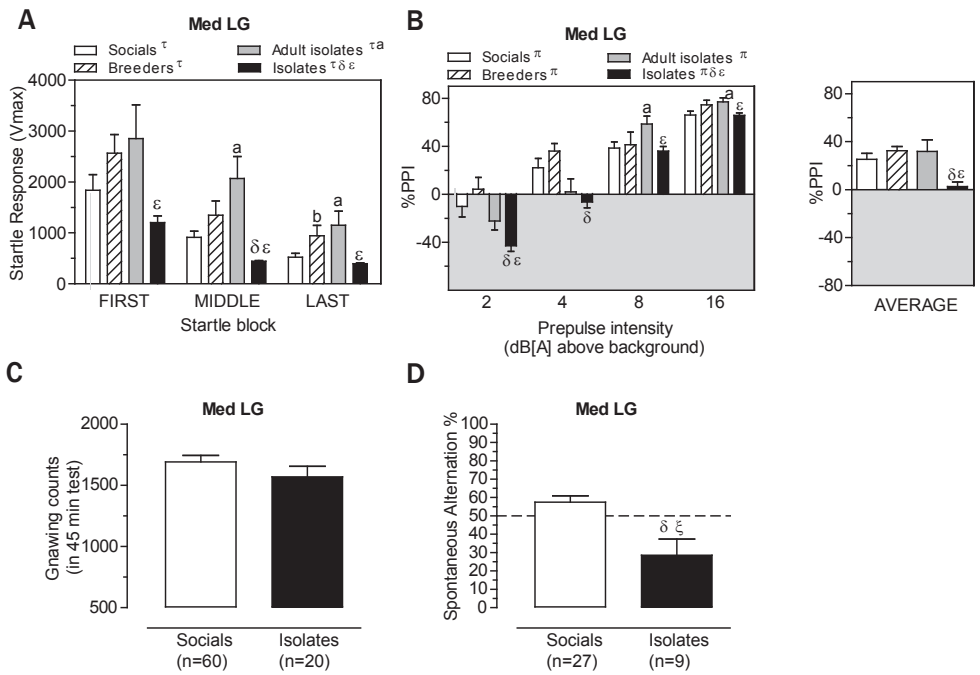

Figure S6. Acoustic startle (A), Prepulse inhibition (B), apomorphine induced gnawing (C), T-maze short-term memory (D) of Med LG APO-SUS rats housed in different post-waning social environments. Left panel B show PPI expressed per prepulse intensity level, whereas right panel B show PPI data expressed as average across all prepulse intensities. Data presented as MEAN ± SEM. a adult isolates vs. corresponding values of socials, b breeders vs. corresponding values of socials, δ isolates vs. corresponding values of socials, ε isolates vs. corresponding values of adult isolates, τ denotes time effect, π denotes prepulse intensity effect, ξ vs. 50% (chance level).

Table S1.

Body weight in gr of WH and APO-SUS rats (Experiment I&II)

pnd	1	11	21	46	53	60	67	74	81	88	95	102	109	116	165	179
WH	5.92 ± 0.10	22.98 ± 0.18	48.28 ± 0.30	196.66 ± 2.46	238.53 ± 1.86	276.00 ± 4.73	319.75 ± 5.78	330.68 ± 6.04	348.42 ± 3.82	376.25 ± 4.39	387.65 ± 6.71	399.03 ± 3.72	410.08 ± 4.33	424.00 ± 5.36	452.03 ± 9.21	472.08 ± 13.32
APO-SUS	6.16 ± 0.08	21.39 ± 0.17 *	40.21 ± 0.32 *	164.52 ± 3.44*	201.69 ± 2.15*	232.25 ± 4.50*	263.50 ± 3.66*	281.20 ± 3.00*	293.83 ± 2.95*	297.67 ± 8.29*	335.84 ± 6.71*	336.47 ± 4.04*	341.87 ± 4.00*	342.22 ± 7.71*	402.55 ± 6.15*	406.20 ± 9.40*

One-way ANOVA on body weight revealed significant effects of rat strain for postnatal day (pnd) 11 ($F_{1,294}$=33.816; p<0.001), pnd 21 ($F_{1,266}$=317.261; p<0.001), pnd 46 ($F_{1,56}$=60.895; p<0.001), pnd 53 ($F_{1,154}$=138.761; p<0.001), pnd 60 ($F_{1,33}$=36.577; p<0.001), pnd 67 ($F_{1,15}$=67.603; p<0.001), pnd 74 ($F_{1,46}$=46.521; p<0.001), pnd 81 ($F_{1,85}$=75.542; p<0.001), pnd 88 ($F_{1,21}$=82.618; p<0.001), pnd 95 ($F_{1,43}$=15.390; p<0.001), pnd 102 ($F_{1,99}$=99.351; p<0.001), pnd 109 ($F_{1,75}$=96.614; p<0.001), pnd 116 ($F_{1,27}$=75.282; p<0.001), pnd 165 ($F_{1,38}$=12.357; p=0.001) and pnd 179 ($F_{1,20}$=12.507; p=0.002). Data presented as MEAN ± SEM. * vs. corresponding values of WH.

Table S2.

. Body weight in gr of APO-SUS socials (Experiment III)

pnd	1	11	21	53	74	81	102	109	165
High LG	5.98 ± 0.05	21.82 ± 0.33	40.83 ± 0.56	209.47 ± 3.96	288.25 ± 14.98	320.33 ± 6.18	346.00 ± 4.94	350.67 ± 3.17	412.17 ± 9.94
Low LG	6.06 ± 0.18	20.88 ± 0.27$^{\Psi}$	38.36 ± 0.64 $^{\Psi}$	197.79 ± 1.97 $^{\Psi}$	244.33 ± 11.45 $^{\Psi}$	290.50 ± 1.63 $^{\Psi}$	331.75 ± 3.94 $^{\Psi}$	333.29 ± 6.47 $^{\Psi}$	388.44 ± 4.55 $^{\Psi}$

One-way ANOVA on body weight revealed significant effects of maternal care history for postnatal day (pnd) 11 ($F_{1,41}$=4.435; p=0.042), pnd 21 ($F_{1,28}$=8.145; p=0.008), pnd 53 ($F_{1,35}$=6.913; p=0.013), pnd 74 ($F_{1,15}$=12.123; p<0.001), pnd 81($F_{1,15}$=21.760; p<0.001), pnd 102 ($F_{1,16}$=5.190; p=0.038), pnd 109 ($F_{1,15}$=5.818; p=0.030) and pnd 165 ($F_{1,19}$=4.696; p=0.044). Data presented as MEAN ± SEM. ψ vs. corresponding values of High LG.

Table S3.

Body weight in gr of Med-LG APO-SUS offspring (Experiment IV)

pnd	1	11	21	46	53	81	109	116
Socials	6.29 ± 0.09	21.37± 0.23	40.65 ± 0.56	166.35 ± 3.58	202.23 ± 1.93	296.27± 4.25	344.46 ± 5.72	345.57 ± 6.57
Isolates	-	-	40.87± 0.79	182.00 ± 6.65 $^{\delta}$	213.54 ± 4.25 $^{\delta}$	288.20 ± 6.90	351.38 ± 9.57	354.88 ± 8.92

One-way ANOVA effect on body weight revealed significant effects of the post-weaning rearing environment for postnatal day (pnd) 46 ($F_{1,24}$=5.138; p=0.033) and pnd 53 ($F_{1,60}$=7.148; p=0.010). Data presented as MEAN ± SEM. δ isolates vs. corresponding values of socials.

Table S4..

Body weight in gr of APO-SUS isolates (Experiment V)

pnd	1	11	21	53	95	109	116	165
High LG	5.98 ± 0.05	21.82 ± 0.33	40.56 ± 0.29	208.57 ± 3.96	334.00 ± 1.89	354.67 ± 1.83	360.00 ± 1.83	405.33 ± 3.57
Low LG	6.06 ± 0.18	20.88 ± 0.27$^{\Psi}$	39.11 ± 0.45$^{\Psi}$	219.83 ± 3.63	325.75 ± 4.04	347.50 ± 6.47	348.75 ± 4.02$^{\Psi}$	392.00 ± 5.09 $^{\Psi}$

One-way ANOVA on body weight revealed significant effects of maternal care history for postnatal day (pnd) 11: ($F_{1,41}$=4.435; p=0.042), pnd 21 ($F_{1,41}$=7.116; p=0.017), pnd 116 ($F_{1,17}$=5.757; p=0.029), pnd 165 ($F_{1,17}$=4.603; p=0.048). Data presented as MEAN ± SEM. ψ vs. corresponding values of High LG.

S3. Supplementary References

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