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Inter-α-inhibitor deficiency in the mouse is associated with alterations in anxiety-like behavior, exploration, and social approach

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Abstract

In recent years, several genome-wide association studies have identified candidate regions for genetic susceptibility in major mood disorders. Most notable are regions in a locus in chromosome 3p21, encompassing the genes *NEK4-ITIH1-ITIH3-ITIH4.* Three of these genes represent heavy chains of the composite protein inter-α-inhibitor (Iα1). In order to further establish associations of these genes with mood disorders, we evaluated behavioral phenotypes in mice deficient in either *Ambp/bikunin,* which is necessary for functional ITIH1 and ITIH3 complexes, or in *Itih4,* the gene encoding the heavy chain Itih4. We found that loss of *Itih4* had no effect on the behaviors tested, but loss of Ambp/bikunin led to increased anxiety-like behavior in the light/dark and open field tests and reduced exploratory activity in the elevated plus maze, light/dark preference, and open field tests. Ambp/bikunin knockout mice also exhibited a sex-dependent exaggeration of acoustic startle responses, alterations in social approach during a 3- chamber choice test, and an elevated fear conditioning response. These results provide experimental support for the role of ITIH1/ITIH3 in the development of mood disorders.

Introduction

Major mood disorders have a lifetime prevalence of up to 20%, causing significant morbidity (1) and disability (2). Clinical presentation characteristics of these disorders suggest that they are highly heritable traits; however, until recently there was scant concrete evidence for genetic susceptibility factors. In the last few years, several gene-wide association (GWA) studies have provided information on candidate genes that may predispose one to bipolar disorder and/or schizophrenia. Initial findings, that were subsequently replicated, suggested a candidate locus in chromosome 3p21, a region encompassing the genes *NEK4-ITIH1-ITIH3-ITIH4* (3–6). Inter-α-trypsin-inhibitor (ITIH)1, ITIH3, and ITIH4 are members of the inter-a-inhibitor family, which has matrix stabilizing and anti-inflammatory properties (7).

Inter-α-inhibitor heavy chains ITIH1 and ITIH3 exist only in a complex with the light chain protein bikunin, which confers the trypsin-inhibitory activity that gives the protein its name. In humans, the heavy chain-light chain stoichiometry is either 2:1 (classic inter-α-inhibitor protein, comprised of ITIH1, ITIH2 and bikunin, MW 250 kDa) or 1:1 (pre-α-inhibitor protein, comprised of ITIH3 and bikunin, MW 125 kDa). ITIH4 lacks the binding site for bikunin and is found as an isolated heavy chain in serum. The liver is the major expression site for lαl, pre-αl, or ITIH4, and secretes fairly high concentrations (100–500 ¼g/ml) of proteins to the serum in healthy subjects (7). However, heavy chain and bikunin expression occurs in many organs, including the brain (8–11). Interestingly, ITIH3 and ITIH4 were identified as acute phase markers in cerebral ischemia (12,13). Recent work also highlighted salutary effects of lαl in neurological recovery after brain injury (14,15). The involvement of lαl and its heavy chains in brain injury and repair suggests a possible role in maintaining normal homeostatic balance and that genetic polymorphisms in these proteins may contribute to the risk of neuropsychiatric disorders.

In this study, we investigated potential functional effects of candidate genes *ITIH1-ITIH3-ITIH4* on rodent behavior. Because (to our knowledge) there are no *Itih1* or *Itih3* deficient mice, we started by contrasting lαl heavy chains ITIH1 and ITIH3 to ITIH4. We achieved this by examining the behavior of *Ambp* (Alpha-1-microglobulin/bikunin precursor) knockout (KO) mice, which lack the bikunin light chain. Because this light chain is necessary for the assembly of the mature lαl and pre-αl protein, these mice are globally lαl-deficient (16), and ITIH1/ITIH3 should have no function, even though they are still expressed. However, these mice still have a functional ITIH4, since ITIH4 does not form a complex with bikunin. We compared these mice to *Itih4* KO mice, which have functional lαl and pre-αl (i.e. functional ITIH1/ITIH3) proteins but are deficient in ITIH4. We found that genetic deficiency in Ambp (i.e. functional deficiency in lαl and pre- al which contain ITIH1 and ITIH3) was associated with a reduction in exploratory behavior and social novelty preference, elevated freezing behavior in both cued and contextual fear conditioning, and an exaggerated startle response seen only in females. In contrast, ITIH4 deficiency did not have an appreciable effect on mouse behaviors tested. Our results support a role for ITIH1 and/or ITIH3 (as opposed to ITIH4) in the development of mood disorders.

Materials and methods

Animals

Generation of the *Ambp* and *ITIH4* wild type (WT) and KO mice was conducted as described (16,17). Mice had been backcrossed to C57BL/6J (Jackson Laboratories, Bar Harbor, ME) for more than 10 generations and were derived from heterozygous (HET) x HET pairings at NIEHS. At weaning, mice were genotyped, ear-punched for identification, and those randomly selected for behavioral testing were group housed by sex. Behavioral phenotyping was conducted on separate sets of mice at NIEHS [male *Ambp* KO and WT mice or male *Itih4* KO and WT mice randomly selected from 11 litters across 3 cohorts] and at the University of North Carolina (UNO; Chapel Hill, NC) [male and female *Ambp* KO and WT randomly selected from 9 litters] to replicate tests conducted at NIEHS and for additional behavioral tests. At NIEHS, male mice were housed by genotype (3–4 mice per cage; 2 cages of 5 mice) within an Association for Assessment and Accreditation of Laboratory Animal Care international accredited facility. Following transfer to UNO at 6 weeks-of-age, mice were housed with original cage-mates, separated by sex (2–4 per cage). Mice were housed in ventilated cages (Techniplast, Exton, PA) (NIEHS: autoclaved hardwood bedding (Sani-chips, PJ Murphy, Montville, NJ) and autoclaved Enviro-dri® nesting material (Shepherd Specialty Papers, Watertown, TN); UNO: irradiated Bed-o'Cobs bedding (The Andersons, Maumee, OH) and cotton nestlets (Ancare, Bellmore, NY) under a 12:12 hour light:dark cycle (6:00– 18:00 EST) at 22+/−2°C and ≈40% relative humidity. Food (NIEHS: autoclaved NIH31; Harlan Laboratories, Madison, Wl; UNO: Teklad Diet #2020SX, Envigo, Madison, Wl) and reverse-osmosis drinking water were available *ad libitum.* Sentinel mice tested negative for pathogens. Adult body weights did not differ with genotype. All procedures were conducted in compliance with the policies on animal welfare of the National Institutes of Health ("Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, 1996) according to protocols approved by each Institute's Animal Care and Use Committee.

Behavioral Assessments

Mice were semi-randomly assigned to test chambers or test sequence to ensure a balanced distribution of genetic background. Mice were evaluated in only one type of test per day. All assessments were conducted in a manner to maintain experimenter blinding to the mouse genotype. For testing at NIEHS, 12-week-old male *Ambp* mice (WT n=19; *Ambp* KO n=18; 2 cohorts) and *itih4* mice (WT n=18; KO n=18; 3 cohorts) were sequentially assessed for open field exploratory activity, acoustic startle/prepulse startle inhibition (PPI), fear conditioning, and forced swim. For testing at UNC, mice (WT: 6 females; 9 males; *Ambp KO*: 9 females; 6 males) were randomly assigned to 2 cohorts, balanced for genotype and sex. Mice, 7–8 weeks of age, were sequentially for elevated plus-maze and light-dark place preference; open field activity (8–9 and 23–24 weeks of age); rotarod (9–10 weeks of age); sociability and social novelty (10–11 weeks of age); marble burying (12–13 weeks of age); startle response and PPI (12–13 and 17– 18 weeks of age); contextual and cued fear conditioning (14–15 weeks of age) memory retention (16–17 weeks of age); forced swim (17–18 weeks of age); and amphetamine- induced hyperactivity (24–25 weeks of age).

Test Methods

Elevated Plus-maze.: The plus-maze (two open arms (8 × 30 cm); two closed arms (8 × 30 cm x 20 cm)), was elevated 50 cm from the floor. Individual mice were placed on the center section (8 cm x 8 cm) and allowed to freely explore the maze for 5-min. Entries into and time spent in each arm were recorded during the 5-min trial by a human observer.

Light/dark Preference Test.: The photocell-equipped open-field arena within a sound-attenuated chamber was divided in half by black Plexiglas inserts. One side was a dark enclosure, the other side was lighted with access by small opening. Mice were placed in the lighted side and the time spent in the lighted side, entries into the lighted side, and total entry number (crosses between sides) were recorded for 10 min.

Open-field Activity.: Exploratory activity in a novel environment was assessed in a photocell-equipped open-field an arena (40 cm x 40 cm x 30 cm) (NIEHS: OptoMax Activity, Columbus Instruments, Columbus, OH; UNC: Versamax, Accuscan Instruments, Columbus, OH) configured in 16 × 16 grids. Photobeam configuration for ambulatory activity at NIEHS (2.5 cm above floor; 1.27 cm apart) was comparable to configuration at UNC (1.5 cm above floor; 2.5 cm apart). UNC configuration additionally accessed rearing behavior with photocells placed at 7 cm above floor. A 25 cm x 25 cm center region was defined. For NIEHS a 1 photocell margin region was defined, in 5- min epochs over 30 min. In 5-min epochs over 30 or 60 min, total distance traveled in the arena, distance traveled and time spent in the center were recorded. Time spent in the margin of the arena was recorded at NIEHS and rearing events were recorded at UNC.

Rotarod.: Balance and motor coordination were assessed on an accelerating (3 – 30 rpm) rotarod (Ugo-Basile, Stoelting Co., Wood Dale, II). Mice were given 3 consecutive trials with a 5-min maximum per trial, and with a 45 sec ITI. Forty-eight hours later, mice received 2 additional trials. Latency to fall was recorded.

Sociability and Preference for Social Novelty.: Social approach was assessed using a three-chambered Plexiglas box (18,19) (40 cm x 60 cm x 20 cm), with retractable doorways allow entry into the side chambers. The test had three consecutive 10-min phases: habituation, sociability, and preference for social novelty. Habituation: the mouse was placed in the middle chamber of the empty test box. The doorways were opened for free exploration over 10 min. Sociability: the mouse was enclosed in the center compartment. An unfamiliar stranger (stranger 1, a sex-matched C57BL/6J adult mouse) was placed in Plexiglas cage (10cm x 10 cm x 20 cm; with 1 cm air holes) in one side-chamber, while an identical empty cage was placed in the other side-chamber. The location of stranger 1 alternated between the left and right chambers of the social test box across subjects. The doors were opened and total time spent within 5-cm proximity to each Plexiglas cage and number of entries into each side were recorded by an image-tracking system (Ethovision, Noldus Information Technology, Wageningen, the Netherlands). Social novelty preference: the mouse was enclosed in the center chamber and a new unfamiliar mouse (stranger 2 from a home cage different from that of stranger 1) was placed into the previously empty Plexiglas cage. The

doors to the side chambers were opened and proximity time and number of entries over 10 min recorded.

Marble-burying Assay.: Twenty black glass marbles (14 mm) were arranged in a equidistant 5 × 4 array on top of 5-cm deep, clean corncob bedding in a clean polycarbonate cage (20 cm x 36 cm x 13 cm) placed in a sound-attenuating chamber with ceiling light and fan. The number of marbles covered 2/3rd or more by the bedding within 30 min was recorded.

Acoustic Sstartle and Prepulse Startle Inhibition (PPI).: Auditory startle response, habituation, and prepulse-startle inhibition (PPI) as a measure of sensorimotor gating were assessed (SR-Lab startle apparatus; San Diego Instruments, San Diego, CA). Mice were placed within a cylinder holder and allowed a 5-min habituation period. At NIEHS the session consisted of 6 pretest 120 dB trials (pulse-alone) followed by a main test unit of 52 trials presented at 15-sec variable inter-trial intervals (ITI; 10–20 sec), followed by 6, 120 dB post-test trials. Background levels of 70 dB were maintained. Main test trials were comprised of two 26 trial-blocks: 1) no-stimulus trials, 2) acoustic startle stimulus trials (40-msec null period followed by 40-msec 120 dB pulse), and 3) prepulse stimulus trials (40-msec null period followed by 20-msec pre-pulse of 3, 6, or 12 dB above background [73, 76 and 82 dB] followed by a 100-msec null period and a 40- msec 120dB pulse) presented in a randomized order within each block. The prepulse+pulse trial recording period was 200 msec. Startle reactivity (median response to 120dB pulse trials (80 msec recording window), % habituation (change from 1st block of trials), and prepulse startle inhibition (%PPI) for the 3, 6, and 12-dB prepulse+pulse trials were determined. %PPI was calculated as [(median Vmax of 120dB pulse trials- median pre-pulse Vmax response)/ median Vmax of 120dB pulse trials] x 100. At UNC the session consisted of 7 blocks of 7 different randomized trial types: no-stimulus trials, trials with the 120 dB startle stimulus (40 ms) alone, and pre-pulse trials (20 ms; either 74, 78, 82, 86, or 90 dB presented 100 ms before the 120 dB startle stimulus), with a 15 sec variable ITI. Background level of 70dB and a 65-msec sampling window were maintained. The peak startle response and mean PPI, (100 - [(response amplitude for pre-pulse stimulus and startle stimulus together / mean response amplitude for startle stimulus alone) x 100]) were calculated for entire session.

Contextual and Cued Fear Conditioning.: At NIEHS, training and testing procedures were performed using a modified Gemini shuttle-box apparatus (San Diego Instruments, Inc.) comprised of two compartments separated by a closed guillotine door with matched lighting, cue light, and tone delivery and a stainless steel grid floor. At UNC, mice were evaluated using the Near-Infrared image tracking system (MED Associates, Burlington, VT), comprised of Plexiglas test chambers (28 cm x 33 cm x 25 cm) housed in a sound attenuating chamber. At both locations, on training day 1, the mouse was allowed 2-min exploration in test chamber. A 30-sec 80dB tone and cue light were co-terminated with a 2-sec scrambled foot shock (0.4 mA). Mice received 2 additional shock-tone pairings (NIEHS; 120 sec ITI; UNC: 80 sec ITI). Context-dependent learning was evaluated 24- hr later in the original test chamber. At NIEHS the latency to first freezing event and duration of freezing were recorded over a 3-min session by 2 independent observers. At UNC, the % time

immobile across a 5-min session was determined by automated tracking system. Associative learning was assessed 4-hr later at NIEHS, or at 24-hr later at UNC using a modified chamber (solid floor, chamber shape and texture, novel vanilla odor). During a 2-min exploratory activity interval, levels of freezing were recorded. Associated cues were delivered for 3-min and the levels of freezing recorded. At NIEHS, freezing was recorded for 30-sec (cohorts 1 & 2 *Ambp* mice) or 3-min interval (cohort 2; *Ambp* mice (n=10); Cohorts 1,2,&3; *Itih4* mice). At UNC, after a 2-wk interval, a contextual-fear retention trial was conducted followed 24-hr by a cued-fear trial.

Forced Swim.: Mice were placed in a clear cylinder filled with 12–15 cm of clean tap water (23–26°C) and swimming behavior video-tracked for 6 min (NIEHS: Any-Maze, Stoelting Co; UNC: Noldus Ethovision, Wageningen, The Netherlands). Latency to the first episode of immobility and total time spent immobile or swimming during the final 4 min of the session were recorded.

Amphetamine Challenge.: According to a split-plot design, 1/2 of each group received an intraperitoneal injection (1 ml/100g body wt) of either saline or d-amphetamine sulfate (2 mg /kg body wt; Millipore Sigma, St. Louis, MO). Mice were immediately placed in the open-field activity arena to record ambulatory and rearing activity in 5 min epochs for 150 min. Animals were semi-randomly assigned to order of injection and balanced across genotype and home cage. After a 1-wk drug clearance interval, the vehicle or drug delivery was reversed.

Statistical Analysis.: Data obtained from NIEHS cohorts were tested for homogeneity of variance using Levene's tests and for non-normality using Shapiro-Wilk tests. Motor activity measures of distance travelled were log-transformed and linear mixed-effects models fit as a function of time (5-min epochs) and genotype (Pinheiro and Bates, 2000). Linear mixed-effects models accounted for autocorrelated errors for each animal, separate variance terms for genotype, and random intercepts and slopes to reflect between-animal variability in initial activity and trend over time. One outlier Ambp KO mouse was excluded due to extreme activity levels and one *Itih4* mouse was excluded due to margin photocell failure. Similar linear mixed-effects models were fit to center and margin time. Center time of *Ambp* KO mic was log-transformed. Genotype differences were evaluated by Wald tests. Startle habituation [difference between median 120 dB pulses in 1^{st} and 4^{th} block], startle amplitude (Vmax) for each prepulse level, and %PPI were analyzed by Wilcoxon rank sum tests. Forced swim latency to first immobility bout and duration of immobility were analyzed by Kruskal Wallis rank sum tests. For fear conditioning, a linear mixed-effects model was fit with genotype and age as a linear term and litter nested within each genotype as a random term and Wald tests performed. All models accounted for cohort. Statistical significance was set at $p < 0.05$.

Data obtained from UNC were analyzed for effects of genotype, pharmacological treatment, and time using one-way, two-way, or RM-ANOVA. Separate body weight analyses were conducted for males and females and sex was a factor in the analysis of acoustic startle magnitude (20). Independent group means comparisons were conducted by Fisher's protected least-significant difference (PLSD) tests. Within- genotype comparisons were used

to determine side preference in the 3-chamber test for social approach. Statistical significance was set at $p < 0.05$. All figures, unless otherwise noted, represent pooled populations of males and females.

Results

NIEHS Cohorts

Open Field Activity.—Activity as measured by total distance travelled in the arena showed an expected decrease over the 30-min session (Fig. 1A; $F_{(1,179)}=130.18$, $p<0.0001$). Activity in the entire arena was lower by 12% (95% Cl (3.5%, 20.5%) in *Ambp KO*, compared to WT (Fig. 1A; $F_{(1,34)}=7.59$, $p=0.009$). Activity in the center of the arena was 29% lower in the *Ambp KO* mice, compared to WT (Fig. 1B; $F_{(1,34)}=8.162$, $p=0.007$). *Ambp KO* mice spent 28% less time within the center of the arena (Fig 1C; $F_{(1,34)}=9.23$, $p=0.0045$) and 19% more time within the margin (Fig. 1D; $F_{(1,29)}=14.46$, $p=0.0007$), compared to WT. No significant differences were observed in *Itih4 KO* mice, compared to WT (Fig. 2A-D).

Startle and PPI.—No significant differences were observed in acoustic startle response or startle habituation in *Ambp* KO, compared to WT (Fig. 1E). A significant difference in PPI was observed across pre-pulse startle intensities with no statistically significant difference observed across genotypes (Fig. 1F). No differences were seen between *Itih4 KO* and WT (Fig. 2E-F).

Fear Conditioning.—In both contextual and cued fear conditioning assessments, no difference was observed between *Ambp KO* and WT mice for latency to first freeze event (Fig. 1G). In cued fear conditioning, the percent time freezing (Fig. 1H) was significantly longer in *Ambp KO* mice by 4.12 sec (95% Cl (1.48; 6.75) at the 30-sec interval $F_{(1,15)} = 9.831$, $p=0.006$) and by 21.90 sec (95% (6.88; 36.92) at the 3-min interval ($F_{(1,9)}= 9.789$, $p=0.012$), compared to WT. In contextual fear conditioning (Fig. 11) the freezing response of *Ambp KO* mice was 15.64% (95% Cl (7.20%, 24.08%)) greater, compared to WT ($F_{(1,14)}=12.75$, $p=.003$). No differences were observed between *Itih4 KO* and WT (Fig. 2G-I).

Forced Swim.—No significant differences were observed between *Ambp* KO or *Itih4* KO and respective WT in latency to first immobility event (Fig. 1J; Fig. 2J) or duration of immobility (Fig. 1K; Fig. 2K). This finding was replicated at UNC in immobility durations *(Ambp* KO: 200 sec ± 7; WT: 210 sec ± 5 (mean +/− SEM)).

UNC Cohorts

Elevated Plus-maze and Light/dark Preference.—These assessments are based on a natural tendency of mice to actively explore a new environment versus a fear of an open area. In the elevated plus-maze, percentage of open-arm time and entries were similar between *Ambp KO* and WT mice (Table 1). *Ambp* KO mice made significantly fewer total number of arm entries as compared to WT mice [$F_{(1,28)}=9.45$, $p=0.004$]. In light/dark place preference test, *Ambp* KO mice showed a significant decrease in percent time spent in the

lighted section [$F_{(1,28)}$=4.26, p=0.048] and a lower number of chamber entries [$F_{(1,28)}$=4.31, p=0.047], compared to WT.

Open-field Activity.—*Ambp KO* mice showed less distance traveled as compared to WT mice during the first 5 min of the 1st session [genotype x time: $F_{(11,308)}$=1.84, p=0.047] (Fig. 3A) and2nd session [genotype: $F_{(1,28)}$=4.53, p=0.042] (Fig. 3B). In line with the lower levels of locomotor activity, distance travelled in the center of the arena was significantly less in the *Ambp KO* mice as compared to WT mice [Test 1, $F_{(1,28)}$=4.28, p=0.048; Test 2, $F_{(1,28)}$=7.46, p=0.011] (Table 1). No differences were observed in time spent within the center (data not shown). The additional evaluation of rearing showed a significant overall decrease in *Ambp KO* mice in the 1st [$F_{(1,28)}$=8.07, p=0.008] and 2nd session [$F_{(1,28)}$=10.13, p=0.003] (Fig. 3C and D) as compared to WT mice. *Rotarod Test.* During the initial learning in the rotarod task, the *Ambp* KO mice had shorter latencies to fall than WT (Fig. 3E). A RM-ANOVA indicated a significant main effect of genotype [$F_{(1,28)}$=5.61, p=0.025] and genotype x trial interaction [$F_{(2,56)}$=3.31, p=0.043] on the 1st day that were no longer present in the 2nd test, suggestive of a selective effect on acquisition but not general motor ability.

Social Approach.—All mice showed a significant preference for spending time in proximity to the stranger mouse versus the empty cage [$F_{(1,28}$=94.66, p<0.0001] however, *Ambp* KO mice spent longer time in proximity to the stranger mouse, in contrast to WT [$F_{(1,28)}$=6.34, p=0.0178] (Fig. 3F). In the social novelty test, *Ambp* KO mice showed less shift in preference to the newly-introduced second stranger, in contrast to WT mice [$F_{(1,28)}$=9.13, p=0.0053] (Fig. 3G). As an index of exploratory activity, *Ambp* KO mice made significantly fewer entries into the empty cage side than WT mice [$F_{(1,28)}$=9.48, p=0.0046], *Ambp* KO mice demonstrated a preference for entries into the stranger side, versus the empty-cage side [$F_{(1,28)}$=12.1, p=0.0017] that was not observed in WT mice (Fig. 3H). No differences were observed in in number of entries during the test for social novelty preference (Fig. 3I).

Marble-burying Assay.—No significant differences were observed between *Ambp* KO and WT mice in the number of marbles buried over the test session [$F_{(1,28)}$=3.82, p=0.061]. The means (± SEM) of number of buried marbles for each group were 17 ± 0.5 for WT, and 15 ± 1.0 for *Ambp* KO mice.

Acoustic Startle and PPI.—An initial RM ANOVA of startle response amplitude revealed significant genotype x decibel level interactions for both the 1st test [$F_{(6,156)}$=2.52, p=0.0237] (Fig. 4A; 4B) and the 2nd test [$F_{(6,156)}$=3.08, p=0.0071] (Fig. 4C; 4D). Significant genotype x sex interactions were found for both tests [Test 1, $F_{(6,156)}$=5.12, p<0.0001; Test 2, $F_{(6,156)}$=5.07, p<0.0001]. Female *Ambp* KO mice showed exacerbated startle responses as compared to female WT mice. No differences were found for males. Both the WT and *Ambp* KO mice demonstrated similar, robust prepulse inhibition (Fig. 4E; 4F).

Fear Conditioning.—No significant differences were observed between *Ambp* KO and WT mice in the 1st context-dependent and cue-dependent tests (Fig. 5, A-C) and retention trial.

Amphetamine Challenge.—*Ambp KO* and WT mice displayed similar levels of motor activity in an open field following d-amphetamine (2.0 mg/kg) injection (Fig. 5D and E).

Discussion

Research into the possible genetic causes of major mood disorders is still in its early stages. A broad-scale GWA study identified a polymorphism of *ITIH3* as a risk allele for several psychiatric conditions (21). Other genetic analyses have found associations between the locus for *ITIH1/3/4* and schizophrenia, bipolar disorder, and depression (3,4,22,23). It is important to differentiate which ITI heavy chain is involved in the pathogenesis of mood disorders, because they are not interchangeable - on the contrary, they have distinct associations and functions. In humans, ITIH1 is linked with ITIH2 (another heavy chain, encoded on a different locus) and the light chain bikunin, and forms the 250 kDa protein IαI; ITIH3 just binds bikunin and forms the 125 kDa protein pre-al; while ITIH4 does not associate with bikunin at all, and instead exists as an isolated protein. This leads to different activities in health and disease. For example, only IαI/pre-αl associated proteins (i.e. ITIH1 and ITIH3) would be able to bind and stabilize hyaluronan in the extracellular matrix, because bikunin is necessary for this action (16,24,25). Both bikunin and the heavy chains are also necessary for complement inhibition by lαl/pre-αl (26,27). On the other hand, ITIH4 has been shown to regulate IL-6 expression and play a role in liver development and cancer generation (17), something that has not been described for lαl/pre-αl.

In the present study, we found that global loss of lαl/pre-al in mice led to specific behavioral phenotypes associated with anxiety-related behaviors and social approach that reflect symptoms observed in psychiatric disorders (28). These changes occurred in the absence of alterations in PPI, which is used to model the deficits in sensorimotor gating found in schizophrenia (29). In examination of behaviors related to mood and fear (anxiety), our results showed that the targeted disruption of *Ambp* led to a shift in behaviors in the light/dark test, open field, and elevated plus maze, suggestive of a decrease in exploratory behavior that has often been implicated as reflective of anxietylike behaviors. Although the findings from the open field were replicated at both locations, these tests at UNC began within 1–2 weeks of transfer thus, the influence of such transfer on anxiety-related behaviors cannot be excluded when interpreting this data. While a deficit in the exploratory aspects of the behaviors was demonstrated, our data also suggested the possibility of a deficit in motor performance with lower activity levels, and decreases in rearing and rotarod performance that could have influenced the outcome on the various exploratory measures. In motor activity assessments, *Ambp* KO mice showed lower exploratory activity levels yet the general distribution of activity within the margin versus the center of the arena was not suggestive of an anxiety-related response to the novel open environment. In the present study, exaggerated startle amplitudes were observed in female *Ambp* KO mice that were not observed in males. Increased startle responses have been observed in many human disorders related to anxiety, including posttraumatic stress disorder (PTSD), panic disorder, and obsessive- compulsive disorder (30–32). In rodent models of PTSD, exposure to foot-shock or other aversive stressors has been used to induce persistent effects on behavior related to human symptoms, including exaggerated startle responses (33–35). Alternatively, the single observation of an exaggerated startle response in female mice likely represents a specific

effect upon this reflex response rather than a reflection of an altered anxiety response. No differences were observed for PPI which has been a hallmark in animal studies for translation to human schizophrenia, suggesting that the normal sensorimotor gating associated with inhibition is not altered by the deletion of *Ambp.*

The strongest evidence for an anxiety-related behavioral difference was found in fear conditioning. Fear conditioning is cautiously associated with anxiety-related disorders with a meta-analysis of human studies showing a modest increase in acquisition of fear learning of single-cue paradigms (36). The combined group of male and female mice examined at UNC did not demonstrate changes in either contextual or cued fear conditioning for duration of freezing. However, males examined at NIEHS demonstrated an increase in the duration of freezing under both cued and contextual conditions. While the assessment methods differed between automated detection of movement versus observer rating, given the larger group size for the NIEHS cohort, the more likely reason for this difference is based in the difference in behavioral testing experience of the animals. At UNC, the mice underwent a more intensive testing schedule and were older at the time of fear conditioning testing than mice tested at NIEHS which may have influenced performance. In addition, the transfer and shift in housing conditions for mice at UNC could have had a significant impact on the mice in a manner that would mask the findings observed in mice at NIEHS with much less disruption in normal activities.

A remaining question is associated with the mechanistic basis for the behavioral alterations observed with the global loss of lαl or pre-al protein in the *Ambp* KO mice, lαl is an endogenous regulator for systemic inflammatory responses, with a possible immunomodulatory role in the brain (8–10). Dysregulation of immune system function has been implicated in multiple psychiatric disorders, including autism, bipolar disorder, and schizophrenia (37–40). Previous work in mice has shown that gestational exposure to maternal immune activation (MIA) can lead to increased anxiety-like behavior in offspring (41). Prenatal inflammation can lead to an exacerbated startle response in the adult offspring (42) without altering levels of PPI (43). While such a pattern was observed in female *Ambp* KO mice, there was no evidence of an altered startle response in males. Existing literature regarding developmental inflammation does not offer a solid framework for determining potential sex differences. Social behaviors have also been demonstrated to be sensitive to developmental inflammatory status (33,44). In a three- chamber social approach task, *Ambp KO* mice displayed an increase in the duration of time spent in proximity to the stranger mouse that was interpreted as an increase in sociability, but a diminished social novelty preference. Based on reports of such increased sociability in genetic mouse models for autism spectrum disorders and schizophrenia (45–48), this phenotype has been considered as reflective of inappropriate and impulsive social contact. With regards to developmental inflammatory status, exposure of neonatal rats to the pro-inflammatory cytokine, IL-1α resulted in *a* similar increase in sociability (49), while exposure to IL-6 did not alter sociability but did decrease social novelty (44). The current findings suggest an impaired ability in the more complex discrimination between the first stranger and the newly-introduced second stranger in the *Ambp KO* mice. Further, inflammatory challenges have been linked to depression-like behaviors (50,51) and supersensitivity to effects of dopamine

agonists (52,53). However, in the present study, no differences were observed in the forced-swim test or in sensitivity to amphetamine between the WT and *Ambp KO* mice.

The functional effects of lαl/preal may also extend beyond inflammation. As mentioned earlier, these proteins are found in the brain (9), form complexes with the glycosaminoglycan (GAG) hyaluronan and stabilize the hyaluronan extracellular matrix. GAG and proteoglycan (PG) metabolism plays an important role in brain development, and disorders of PG/GAG metabolism have been associated with brain and mental health problems, including schizophrenia and bipolar disorder (54). Overt hyaluronan deficiency causes a reduction in the brain extracellular space, affects neuronal activity and is associated with seizures (55). It is possible that more subtle changes may be caused by lαl/preal deficiency and lead to the observed behavioral changes. It should also be pointed out that *Ambp KO* mice still retain the capacity to express ITIH1 and ITIH3. Although these heavy chains do not have a known function outside the lαl/preal composite protein, it is possible that they retain some part of their functions even in the absence of bikunin, and so lead to an ameliorated behavioral phenotype. In conclusion, our results indicate that lαl/preαl deficiency leads to selective alterations in behavior, and provide preclinical support for published GWAS findings on the association of lαl heavy chains with clinical phenotypes related to mood and anxiety.

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References

1. Ustun TB, Ayuso-Mateos JL, Chatterji S, Mathers C and Murray CJ (2004) Global burden of depressive disorders in the year 2000. Br J Psychiatry, 184, 386–392. [PubMed: 15123501]
2. Kessler RC, Aguilar-Gaxiola S, Alonso J, Chatterji S, Lee S, Ormel J, Ustun TB and Wang PS (2009) The global burden of mental disorders: an update from the WHO World Mental Health (WMH) surveys. Epidemiol Psichiatr Soc, 18, 23–33. [PubMed: 19378696]
3. Hamshere ML, Walters JT, Smith R, Richards AL, Green E, Grozeva D, Jones I, Forty L, Jones L, Gordon-Smith K et al. (2013) Genome-wide significant associations in schizophrenia to ITIH3/4, CACNA1C and SDCCAG8, and extensive replication of associations reported by the Schizophrenia PGC. Molecular psychiatry, 18, 708–712. [PubMed: 22614287]
4. Psychiatric GCBDWG (2011) Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near 0DZ4. Nature genetics, 43, 977–983. [PubMed: 21926972]
5. Schizophrenia Psychiatric Genome-Wide Association Study, C. (2011) Genome-wide association study identifies five new schizophrenia loci. Nature genetics, 43, 969–976. [PubMed: 21926974]
6. Witt SH, Juraeva D, Sticht C, Strohmaier J, Meier S, Treutlein J, Dukal H, Frank J, Lang M, Deuschle M et al. (2014) Investigation of manic and euthymic episodes identifies state- and trait-specific gene expression and STAB1 as a new candidate gene for bipolar disorder. Translational psychiatry, 4, e426. [PubMed: 25136889]
7. Zhuo L and Kimata K (2008) Structure and function of inter-alpha-trypsin inhibitor heavy chains. Connective tissue research, 49, 311–320. [PubMed: 18991084]

8. Chen X, Rivard L, Naqvi S, Nakada S, Padbury JF, Sanchez-Esteban J, Stopa EG, Lim YP and Stonestreet BS (2016) Expression and localization of Inter-alpha Inhibitors in rodent brain. Neuroscience, 324, 69–81. [PubMed: 26964679]

9. Kobayashi H, Sun GW and Terao T (1999) Immunolocalization of hyaluronic acid and inter-alpha-trypsin inhibitor in mice. Cell and tissue research, 296, 587–597. [PubMed: 10370146]

10. Spasova MS, Sadowska GB, Threlkeld SW, Lim YP and Stonestreet BS (2014) Ontogeny of inter-alpha inhibitor proteins in ovine brain and somatic tissues. Exp Biol Med (Maywood), 239, 724–736. [PubMed: 24728724]

11. Takano M, Mori Y, Shiraki H, Horie M, Okamoto H, Narahara M, Miyake M and Shikimi T (1999) Detection of bikunin mRNA in limited portions of rat brain. Life Sci, 65, 757–762. [PubMed: 10466741]

12. Chen R, Vendrell I, Chen CP, Cash D, O'Toole KG, Williams SA, Jones C, Preston JE and Wheeler JX (2011) Proteomic analysis of rat plasma following transient focal cerebral ischemia. Biomark Med, 5, 837–846. [PubMed: 22103620]

13. Kashyap RS, Nayak AR, Deshpande PS, Kabra D, Purohit HJ, Taori GM and Daginawala HF (2009) Inter-alpha-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. Clin Chim Acta, 402,160–163. [PubMed: 19263524]

14. Gaudet CM, Lim YP, Stonestreet BS and Threlkeld SW (2016) Effects of age, experience and inter-alpha inhibitor proteins on working memory and neuronal plasticity after neonatal hypoxia-ischemia. Behavioural brain research, 302, 88–99. [PubMed: 26778784]

15. Threlkeld SW, Gaudet CM, La Rue ME, Dugas E, Hill CA, Lim YP and Stonestreet BS (2014) Effects of inter-alpha inhibitor proteins on neonatal brain injury: Age, task and treatment dependent neurobehavioral outcomes. Exp Neurol, 261, 424–433. [PubMed: 25084519]

16. Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y, Kawamura K, Suzuki T and Kimata K (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. The Journal of biological chemistry, 276, 7693–7696. [PubMed: 11145954]

17. Tang Y, Kitisin K, Jogunoori W, Li C, Deng CX, Mueller SC, Ressom HW, Rashid A, He AR, Mendelson JS et al. (2008) Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling. Proceedings of the National Academy of Sciences of the United States of America, 105, 2445–2450. [PubMed: 18263735]

18. Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP, Barbaro JR, West LM, Threadgill DW and Lauder JM (2007) Mouse behavioral tasks relevant to autism: phenotypes of ten inbred strains. Behav Brain Res, 176, 4–20. [PubMed: 16971002]

19. Nadler JJ, Moy SS, Dold G, Trang D, Simmons N, Perez A, Young NB, Barbaro RP, Piven J and Magnuson TR (2004) Automated apparatus for quantitation of social approach behaviors in mice. Genes Brain Behav, 3, 303–314. [PubMed: 15344923]

20. Duncan GE, Moy SS, Lieberman JA and Roller BH (2006) Effects of haloperidol, clozapine, and quetiapine on sensorimotor gating in a genetic model of reduced NMDA receptor function. Psychopharmacology (Berl), 184, 190–200. [PubMed: 16362405]

21. Cross-Disorder Group of the Psychiatric Genomics, C. (2013) Identification of risk loci with shared effects on five major psychiatric disorders: a genomewide analysis. Lancet, 381,1371–1379. [PubMed: 23453885]

22. Sasayama D, Hori H, Yamamoto N, Nakamura S, Teraishi T, Tatsumi M, Hattori K, Ota M, Higuchi T and Kunugi H (2014) ITIH3 polymorphism may confer susceptibility to psychiatric disorders by altering the expression levels of GLT8D1. JPsychiatrRes, 50, 79–83.

23. Scott LJ, Muglia P, Kong XQ, Guan W, Flickinger M, Upmanyu R, Tozzi F, Li JZ, Burmeister M, Absher D et al. (2009) Genome-wide association and meta-analysis of bipolar disorder in individuals of European ancestry. Proceedings of the National Academy of Sciences of the United States of America, 106, 7501–7506. [PubMed: 19416921]

24. Yingsung W, Zhuo L, Morgelin M, Yoneda M, Kida D, Watanabe H, Ishiguro N, Iwata H and Kimata K (2003) Molecular heterogeneity of the SHAP-hyaluronan complex. Isolation and characterization of the complex in synovial fluid from patients with rheumatoid arthritis. The Journal of biological chemistry, 278, 32710–32718. [PubMed: 12799384]

25. Zhao M, Yoneda M, Ohashi Y, Kurono S, Iwata H, Ohnuki Y and Kimata K (1995) Evidence for the covalent binding of SHAP, heavy chains of inter- alpha-trypsin inhibitor, to hyaluronan. The Journal of biological chemistry, 270, 26657–26663. [PubMed: 7592891]
26. Garantziotis S, Hollingsworth JW, Ghanayem RB, Timberlake S, Zhuo L, Kimata K and Schwartz DA (2007) Inter-alpha-trypsin inhibitor attenuates complement activation and complement-induced lung injury. J Immunol, 179, 4187–4192. [PubMed: 17785858]
27. Okroj M, Holmquist E, Sjolander J, Corrales L, Saxne T, Wisniewski HG and Blom AM (2012) Heavy chains of inter alpha inhibitor (Ialphal) inhibit the human complement system at early stages of the cascade. The Journal of biological chemistry, 287, 20100–20110. [PubMed: 22528482]
28. Powell CM and Miyakawa T (2006) Schizophrenia-relevant behavioral testing in rodent models: a uniquely human disorder? Biological psychiatry, 59,1198–1207. [PubMed: 16797265]
29. Parwani A, Duncan EJ, Bartlett E, Madonick SH, Efferen TR, Rajan R, Sanfilipo M, Chappell PB, Chakravorty S, Gonzenbach S et al. (2000) Impaired prepulse inhibition of acoustic startle in schizophrenia. Biological psychiatry, 47, 662–669. [PubMed: 10745060]
30. Grillon C (2002) Startle reactivity and anxiety disorders: aversive conditioning, context, and neurobiology. Biol Psychiatry, 52, 958–975. [PubMed: 12437937]
31. Grillon C and Baas J (2003) A review of the modulation of the startle reflex by affective states and its application in psychiatry. Clin Neurophysiol, 114, 1557–1579. [PubMed: 12948786]
32. Kumari V, Kaviani H, Raven PW, Gray JA and Checkley SA (2001) Enhanced startle reactions to acoustic stimuli in patients with obsessive- compulsive disorder. Am *J Psychiatry*, 158,134–136. [PubMed: 11136648]
33. Bazak N, Kozlovsky N, Kaplan Z, Matar M, Golan H, Zohar J, Richter- Levin G and Cohen H (2009) Pre-pubertal stress exposure affects adult behavioral response in association with changes in circulating corticosterone and brain-derived neurotrophic factor. Psychoneuroendocrinology, 34, 844–858. [PubMed: 19181453]
34. Pynoos RS, Ritzmann RF, Steinberg AM, Goenjian A and Prisecaru I (1996) A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. Biol Psychiatry, 39,129–134. [PubMed: 8717611]
35. Rasmussen DD, Crites NJ and Burke BL (2008) Acoustic startle amplitude predicts vulnerability to develop post-traumatic stress hyper-responsivity and associated plasma corticosterone changes in rats. Psychoneuroendocrinology, 33, 282–291. [PubMed: 18164825]
36. Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, Grillon C and Pine DS (2005) Classical fear conditioning in the anxiety disorders: a meta-analysis. Behaviour research and therapy, 43,1391–1424. [PubMed: 15885654]
37. Abdallah MW, Larsen N, Mortensen EL, Atladottir HO, Norgaard- Pedersen B, Bonefeld-Jorgensen EC, Grove J and Hougaard DM (2012) Neonatal levels of cytokines and risk of autism spectrum disorders: an exploratory register-based historic birth cohort study utilizing the Danish Newborn Screening Biobank. JNeuroimmunol, 252, 75–82. [PubMed: 22917523]
38. Atladottir H0, Thorsen P, Ostergaard L, Schendel DE, Lemcke S, Abdallah M and Parner ET (2010) Maternal infection requiring hospitalization during pregnancy and autism spectrum disorders. J Autism Dev Disord, 40,1423–1430. [PubMed: 20414802]
39. Brown AS (2012) Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. Dev Neurobiol, 72,1272–1276. [PubMed: 22488761]
40. Marco C, Antonio D, Antonina S, Alessandro S and Concetta C (2016) Corrigendum to “Genes involved in pruning and inflammation are enriched in a large mega-sample of patients affected by schizophrenia and bipolar disorder and controls” [Psychiatry Res 228 (2015) 945–949]. *Psychiatry Res,* 238,165.
41. Hsiao EY, McBride SW, Chow J, Mazmanian SK and Patterson PH (2012) Modeling an autism risk factor in mice leads to permanent immune dysregulation. Proc Natl Acad Sci USA, 109,12776–12781. [PubMed: 22802640]
42. Walker FR, Knott B and Hodgson DM (2008) Neonatal endotoxin exposure modifies the acoustic startle response and circulating levels of corticosterone in the adult rat but only following acute *stress*. J Psychiatr Res, 42,1094–1103. [PubMed: 18406426]

43. Fortier ME, Joober R, Luheshi GN and Boksa P (2004) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. / *Psychiatr Res*, 38, 335–345.
44. Brunssen SH, Moy SS, Toews AD, McPherson CA and Harry GJ (2013) Interleukin-6 (IL-6) receptor/IL-6 fusion protein (Hyper IL-6) effects on the neonatal mouse brain: possible role for IL-6 trans-signaling in brain development and functional neurobehavioral outcomes. Brain, behavior, and immunity, 27, 42–53.
45. Chao HT, Chen H, Samaco RC, Xue M, Chahrour M, Yoo J, Neul JL, Gong S, Lu HC, Heintz N et al. (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature, 468, 263–269. [PubMed: 21068835]
46. Feyder M, Karlsson RM, Mathur P, Lyman M, Bock R, Momenan R, Munasinghe J, Scattoni ML, Ihne J, Camp M et al. (2010) Association of mouse Dlg4 (PSD-95) gene deletion and human DLG4 gene variation with phenotypes relevant to autism spectrum disorders and Williams' syndrome. Am J Psychiatry, 167,1508–1517. [PubMed: 20952458]
47. Moy SS, Ghashghaei HT, Nonneman RJ, Weimer JM, Yokota Y, Lee D, Lai C, Threadgill DW and Anton ES (2009) Deficient NRG1-ERBB signaling alters social approach: relevance to genetic mouse models of schizophrenia. JN*eurodev Disord*, 1, 302–312. [PubMed: 21547722]
48. Moy SS, Nikolova VD, Riddick NV, Baker LK and Roller BH (2012) Preweaning sensorimotor deficits and adolescent hypersociability in Grinl knockdown mice. Dev Neurosci, 34,159–173. [PubMed: 22571986]
49. Tohmi M, Tsuda N, Watanabe Y, Kakita A and Nawa H (2004) Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin. Neurosci Res, 50, 67–75. [PubMed: 15288500]
50. Felger JC and Lotrich FE (2013) Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience, 246, 199–229. [PubMed: 23644052]
51. Miller AH, Maletic V and Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biological psychiatry, 65, 732–741. [PubMed: 19150053]
52. Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H and Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring:a neurodevelopmental animal model of schizophrenia. Biol Psychiatry, 59, 546–554. [PubMed: 16256957]
53. Willi R, Harmeier A, Giovanoli S and Meyer U (2013) Altered GSK3beta signaling in an infection-based mouse model of developmental neuropsychiatric disease. Neuropharmacology, 73, 56–65. [PubMed: 23707483]
54. Maeda N (2015) Proteoglycans and neuronal migration in the cerebral cortex during development and disease. Front Neurosci, 9, 98. [PubMed: 25852466]
55. Arranz AM, Perkins KL, Irie F, Lewis DP, Hrabe J, Xiao F, Itano N, Kimata K, Hrabetova S and Yamaguchi Y (2014) Hyaluronan deficiency due to Has3 knock-out causes altered neuronal activity and seizures via reduction in brain extracellular space. The Journal of neuroscience : the official journal of the Society for Neuroscience, 34, 6164–6176. [PubMed: 24790187]

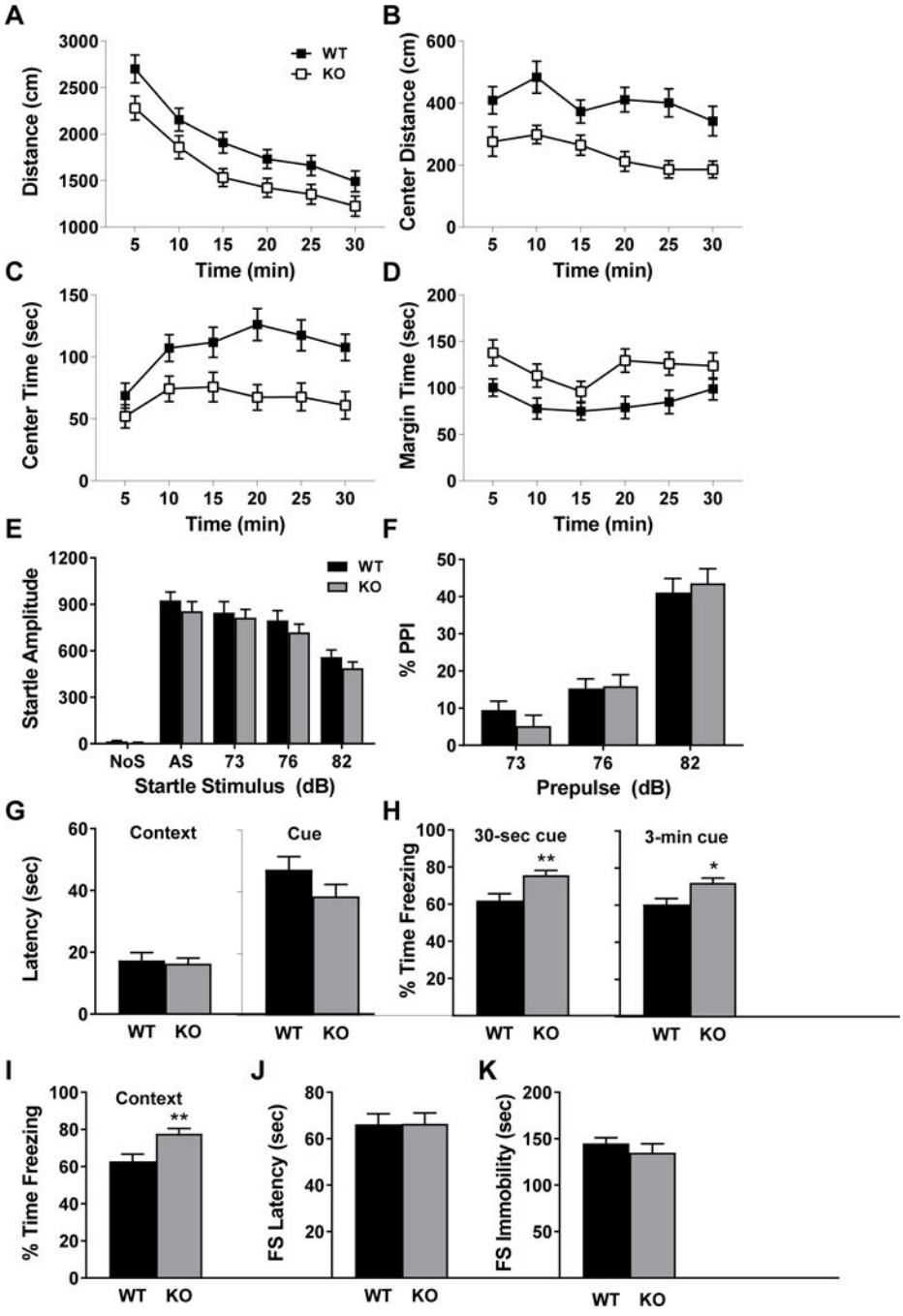

Figure 1.

Behavioral testing conducted at NIEHS in *Ambp KO* and WT adult male mice (A-D) Open field motor activity. Data recorded in 5-min epochs over 30 min. (n=17–19). (A)Distance travelled in the arena significantly decreased over the session ($p<0.0001$). (B)Distance travelled in center. (C) Time spent within the center and (D) margin. (E-F) Acoustic startle response (n=18–19). (E) Startle amplitude (Vmax) at 70 dB background (NoS), 120 (AS), 73, 76, and 82 dB. (F) %pre-pulse startle inhibition (PPI) at each prepulse intensity (73, 76, 82 dB). (G-l) Fear conditioning (n=18–19). (G) Latency to the first freezing event. (H) Cue

testing % time spent freezing during 30 sec and 3 min intervals. (I) Context % time spent freezing. (J-K) Forced swim (n=18–19). (J) Latency to first float event. (K) Time spent immobile. Data represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

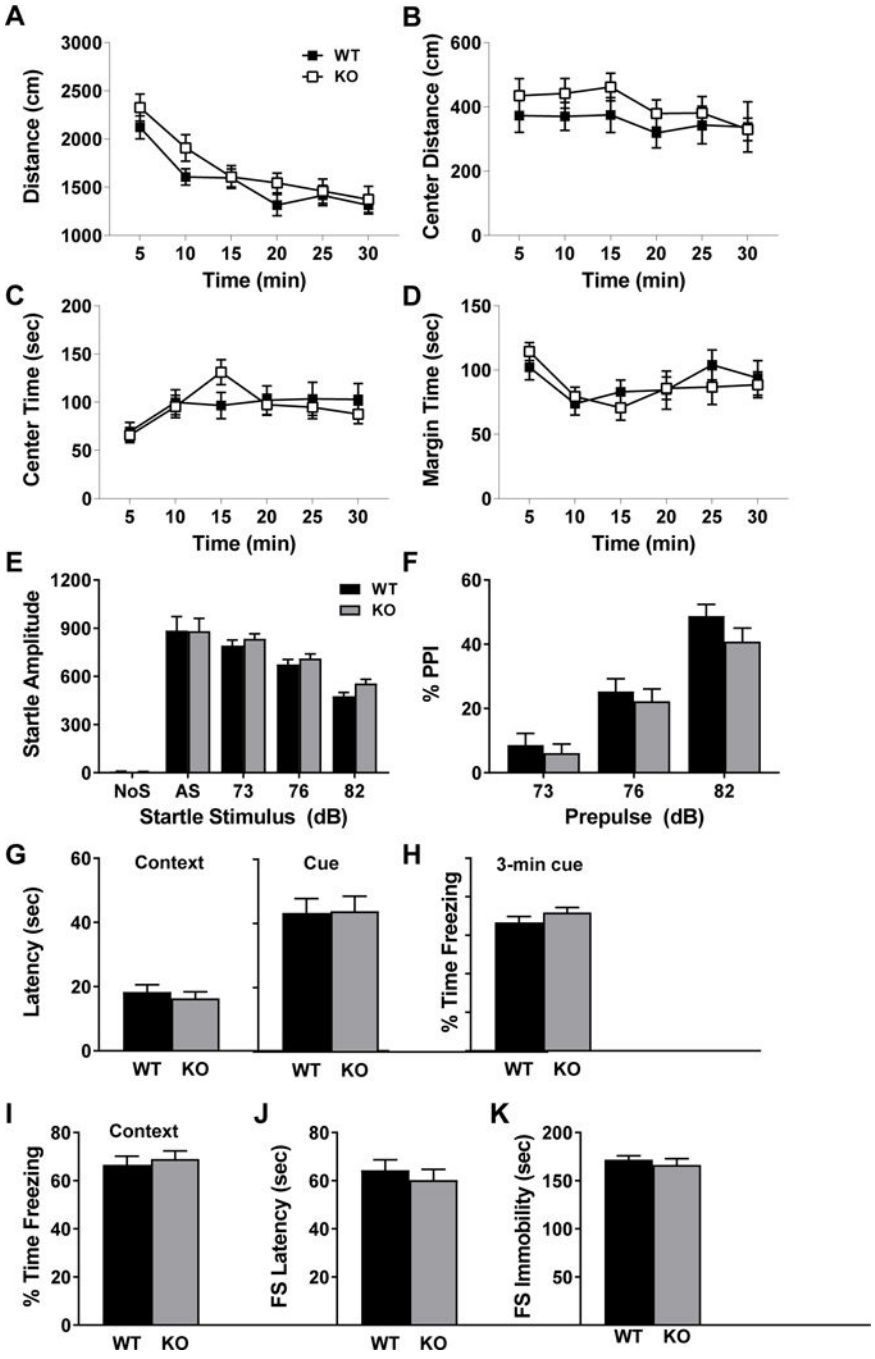

Figure 2.

Behavioral testing conducted at NIEHS in *Itih4* KO and WT adult male mice. (A-D) Open field motor activity. Data recorded in 5-min epochs over 30 min. (n=17–18). (B)Distance travelled in the arena significantly decreased over the session ($p<0.0001$). (B)Distance travelled in center. (C) Time spent within center or (D) margin of the arena. (E-F) Startle response (n=18). (E) Startle amplitude (Vmax) at at 70 dB background (NoS), 120 (AS), 73, 76, and 82 dB. (F) %pre-pulse startle inhibition (PPI) at each prepulse intensity (73, 76, 82 dB). (G-l) Fear conditioning (n=18). (G) Latency to the first freezing event. (H) % time

spent freezing in cue test. (I). % time spent freezing during context conditioning test. (J-K) Forced swim (n=18). (J) Latency to first float event. (K) Time spent immobile. Data represent means ± SEM.

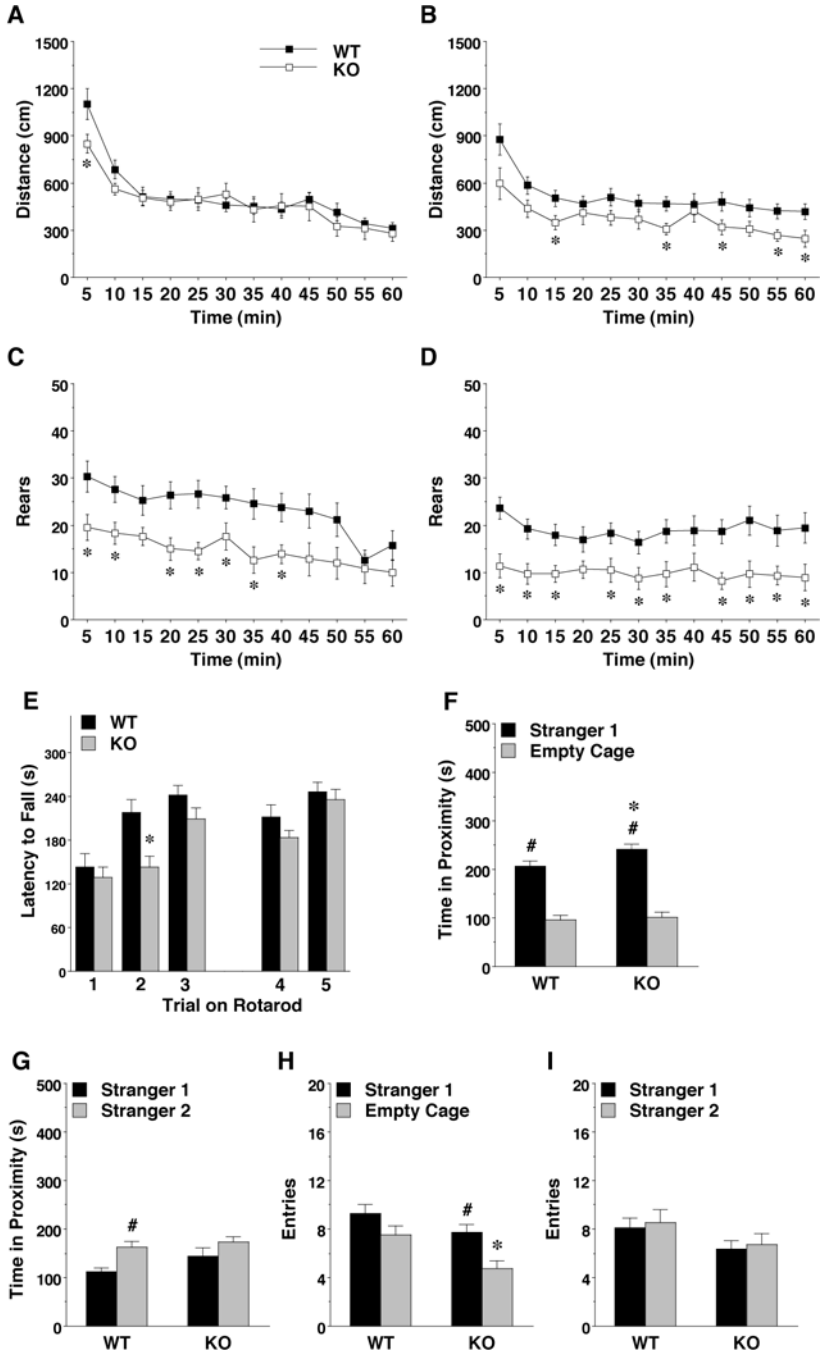

Figure 3.

Open-field activity, rotarod, and social behavior in *Ambp* KO and WT mice tested at UNC. (A-D) Open-field activity in 1 -hr sessions. (A,B) Ambulatory activity during (A) Test 1 (8–9 weeks-of-age) and (B) Test 2 (23 weeks-of-age). (C,D) Rearing activity during (C) Test 1 and (D) Test 2. E) Accelerating Rotarod. Trials 4 and 5 were given 48 hr after first 3 trials. Maximum trial length was 300 sec. (F-l) Social approach in a 3-chamber choice test. Data are mean (+ SEM) for each 10-min test. (F) Social approach (G) Social preference (H) Exploration during test for sociability. (I) Chamber entries during social novelty preference

test. #p < 0.05, within-genotype comparison between stranger 1 side and opposite side. Data represent means (± SEM). *p<0.05, compared to WT.

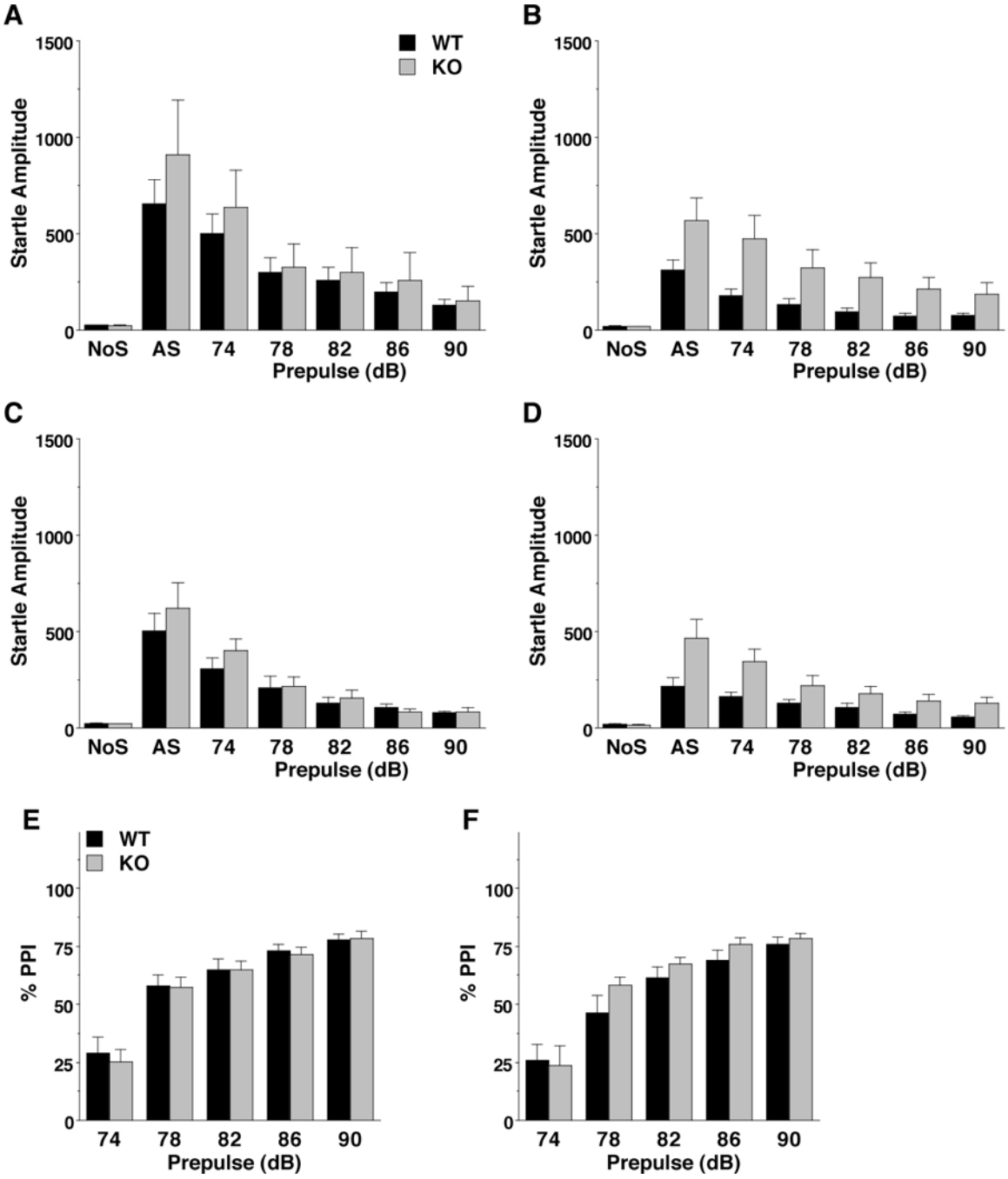

Figure 4.

Startle Reactivity and PPI in *Ambp* KO and WT mice tested at UNC. Trials included no stimulus (NoS) trials and acoustic startle stimulus (AS; 40 ms, 120 dB) alone trials. (A-D) Magnitude of startle responses. (A) Males, (B) Females in Test 1. (C) Males, (D) Females in Test 2. (E-F) Prepulse inhibition in (E) Males or (F) Females in Test 1. Mice were 12–13 weeks-old for Test 1, and 17–18 weeks-old for Test 2. Data represent means (±SEM).

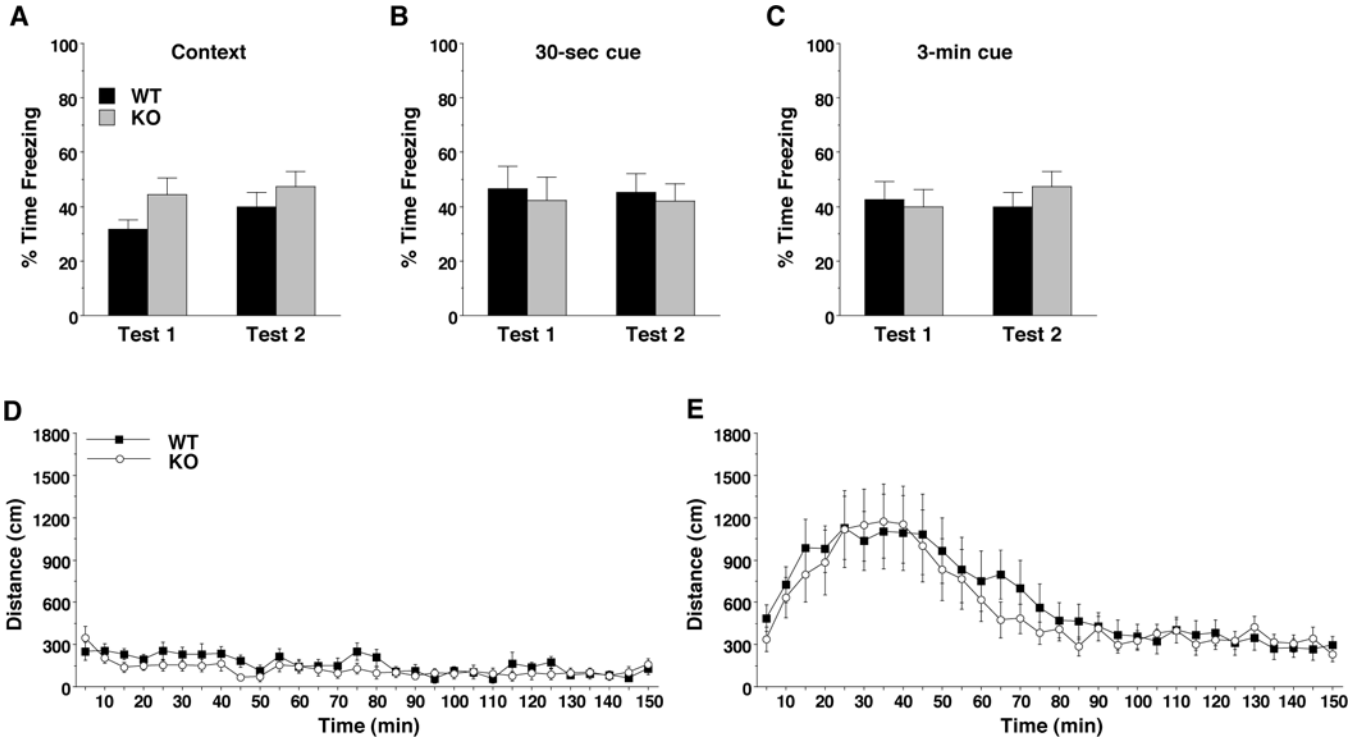

Figure 5.

Conditioned Fear Response and Pharmacological Challenge with d- amphetamine in *Ambp* KO and WT mice. Testing was conducted at UNC. (A-C) Percent time freezing in tests for fear conditioning. Test 2 was conducted two weeks following Test 1. (A) 5-min tests for contextual learning. (B) Freezing during the first 30 s of a tone (80 dB) in the tests for cue-dependent learning. (C) Freezing during the 3- min tone in the tests for cue-dependent learning. Test 1 was conducted in mice 14–15 weeks-old, and Test 2, at 16–17 weeks-old. (D-E) Motor activity stimulant effects of d- amphetamine in a 150-min open-field test. (D) Vehicle. (E) Amphetamine (2 mg/kg,ip). Data represent means (± SEM).

Table 1.

Reduced exploration and increased anxiety-like behavior in *Ambp* KO mice.

	WT	KO
Elevated plus maze		
Percent open arm time	26 ± 3	26 ± 6
Percent open arm entries	34 ± 2	38 ± 4
Total number of entries	25 ± 2	16 ± 2*
Light/dark preference		
Percent time in lighted side	23 ± 3	15 ± 2*
Percent entries to lighted side	37 ± 2	32 ± 2
Total number of entries	56 ± 7	38 ± 6**
Open field		
First test (age 8–9 wk)		
Distance (cm) traveled in center	1457 ± 234	911 ± 122*
Second test (age 23 wk)		
Distance (cm) traveled in center	1522 ± 214	765 ± 176*

Mice were tested at UNC. Data represents means (± SEM).

* $p<0.05$;

** $p<0.01$;