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Behavioral and genetic investigations of low exploratory behavior in *Il18r1*^{-/-} mice: We can't always blame it on the targeted gene

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Abstract

The development of gene targeting technologies has enabled research with immune system-related knockout mouse strains to advance our understanding of how cytokines and their receptors interact and influence a number of body systems, including the central nervous system. A critical issue when we are interpreting phenotypic data from these knockout strains is the potential role of genes other than the targeted one. Although many of the knockout strains have been made congenic on a C57BL/6 (B6) genetic background, there remains a certain amount of genetic material from the 129 substrain that was used in the development of these strains. This genetic material could result in phenotypes incorrectly attributed to the targeted gene. We recently reported low activity behavior in *Il10*^{-/-} mice that was linked to this genetic material rather than the targeted gene itself. In the current study we confirm the generalizability of those earlier findings, by assessing behavior in *Il18*^{-/-} and *Il18r1*^{-/-} knockout mice. We identified low activity and high anxiety-like behaviors in *Il18r1*^{-/-} mice, whereas *Il18*^{-/-} mice displayed little anxiety-like behavior. Although *Il18r1*^{-/-} mice are considered a congenic strain, we have identified substantial regions of 129P2-derived genetic material not only flanking the ablated *Il18r1* on Chromosome 1, but also on Chromosomes 4, 5, 8, 10, and 14. Our studies suggest that residual 129-derived gene(s), rather than the targeted *Il18r1* gene, is/are responsible for the low level of activity seen in the *Il18r1*^{-/-} mice. Mapping studies are necessary to identify the gene or genes contributing to the low activity phenotype.

1. Introduction

Although research with knockout mouse strains plays a critical role in many scientific fields, it is important to remember that any phenotype seen in these animals is not always due to the targeted gene. The nature of the traditional knockout process provides all of the necessary ingredients to give rise to an alternative scenario. When a knockout strain is being developed, the elimination of a functional gene is done either by replacement with a nonfunctional one or by the gene's deletion; these processes are usually performed using embryonic stem cells derived from one of the 129 substrains of mice. Thus, the resulting animals have a 129 genetic background. Due to a general preference by researchers for the C57BL/6J (B6) background,

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the knockout strain is then often crossed to B6, leading to a congenic strain that possesses a section of 129 genome introgressed into a primarily B6 background (Flaherty, 1981; Flaherty and Bolivar, 2007). When the resulting knockout/congenic strain is tested for characteristics that differ between 129 and B6, an ambiguous situation exists, such that any observed phenotype could be due to (i) the targeted gene, (ii) the 129 chromosomal segment flanking the targeted gene, or (iii) other genomic regions that have not converted to B6. This situation is particularly important when the phenotype at issue is one that differs quantitatively between B6 and 129, but is not predicted from the gene expression analyses and/or the known function of the targeted gene. A number of reviews have outlined the confounding factors that are related to the use of knockout/ congenic strains (Crusio, 2004; Eisener-Dorman et al., 2009; Gerlai, 1996; Wolfer et al., 2002), and we have previously demonstrated that low activity in *Il10* null mice is due to flanking-region material rather than to the targeted gene (Rodrigues de Ledesma et al., 2006). Here, we examine the behavioral effects of ablation of the gene that codes either for the proinflammatory cytokine interleukin-18 (IL-18) or for the interleukin18 receptor 1 (IL- 18R1), and we illustrate the importance of genetic background and the need to take this background into account when these behavioral findings are being interpreted.

IL-18 was first identified as interferon-gamma (IFN- γ) inducing factor (Nakamura et al., 1989; Okamura et al., 1995); it is a member of the IL-1 superfamily, sharing structural and functional properties with IL-1 β (Dinarello et al., 1998). Similar to IL-1 β , IL-18 is inflammasome-activated after cleavage by caspase-1 (ICE); non-cleaved pro-IL-18 is inactive as an inflammatory cytokine (Lamkanfi and Dixit, 2009; Martinon et al., 2009). IL-18 is constitutively expressed at a relatively high level in the adult brain and is produced by microglia, neurons, astrocytes, oligodendrocytes, and ependymal cells, under pathophysiological conditions (Cannella and Raine, 2004; Conti et al., 1999; Culhane et al., 1998; Sugama et al., 2002). High brain expression of IL-18 places this cytokine in a key position to quickly respond to and mediate stress, injury, neurological disease, or other CNS-directed insults. In fact, increased levels of IL-18, one of the key mediators of pathological inflammation, may contribute to neurotoxic effects (Felderhoff-Mueser et al., 2005). Brains of Alzheimer's patients display high levels of IL-18 and ICE in the frontal lobe, as well as IL-18 co-localization with amyloid- β plaques and tau protein; IL-18 is also present in multiple CNS resident cells (Ojala et al., 2009). IL-18 production by peripheral blood cells correlates with cognitive decline in Alzheimer's patients (Bossu et al., 2008). Evidence is also accruing that high IL-18 levels impair long-term potentiation (LTP) *in vitro*, and IL-1ra can block the deficit (Cumiskey et al., 2007; Curran and O'Connor, 2001). IL-18, unlike IL-1, does not possess pyrogenic properties; nevertheless, increased brain temperature correlates with intracerebroventricular injection of IL-18 (Kubota et al., 2001).

Ablation of the *Il18* gene results in CNS alterations, notably with respect to infection or other insult. IL-18-deficient (*Il18*^{-/-}) mice with pneumococcal meningitis display decreased neuroinflammation and enhanced survival (Zwijnenburg et al., 2003). They exhibit reduced microglial activation and resistance to hypoxic injury (Hedtjarn et al., 2002; Mori et al., 2001; Sugama et al., 2007; Sugama et al., 2004). They are hyperphagic by early adulthood (Zorrilla et al., 2007), and display reduced rearing in the open field, as well as deficits in spatial learning (Yaguchi et al., 2010). Clearly, IL-18 can be linked to central nervous system function.

The IL-18 receptor (IL18R) is a heterodimer composed of IL18R1 (IL18 α or IL1R5) and IL18R2 (IL18 β or IL1R7). Cleaved IL-18 binds to IL-18R1, which in turn induces signal transduction through IL-18R2 (Arend et al., 2008). In addition to binding IL-18, IL-18R1 binds IL-1F7b, one of the most recently identified members of the IL-1 family (Sharma et al., 2008). An alternate ligand has been suggested to signal through IL-18R1, to induce autoimmune CNS inflammation (Gutcher et al., 2006). The effects of ablation of either of the genes (*Il18r1* and *Il18r2*) responsible for this receptor have not been well studied to date.

Ablation of the *Il18r1* gene has resulted in mice (*Il18r1*^{-/-}) that display defective T-cell-helper type 1 development, a failure to activate NF-κB, and reduced NK cell activity and IFN-γ production (Hoshino et al., 1999). It is not yet clear whether ablation of this gene influences CNS function, either through the IL-18 pathway or via another cytokine pathway. To date, no behavioral abnormalities have been reported in the *Il18r1*^{-/-} knockout strain.

In the present study, we have characterized the behavioral performance of *Il18*^{-/-} and *Il18r1*^{-/-} mice. Importantly, the fact that these strains have been made congenic on a B6 background enables us to separate the effects of residual 129-derived genes and the ablation of the gene of interest. In our investigations of these two mutant strains, we found a low-activity behavioral phenotype in the *Il18r1*^{-/-} mice, but no evidence to support the direct involvement of the targeted gene. The low-activity phenotype of the *Il18r1*^{-/-} mice appears more likely to be related to anxiety than to general motor ability.

2. Methods

2.1 Animals

C57BL/6J (B6), B6.129P2-*Il18*^{tm1Aki}/J (*Il18*^{-/-}; stock 004130) and B6.129P2-*Il18r1*^{tm1Aki}/J (*Il18r1*^{-/-}; stock 004131) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and 129P2/OlaHsd (129P2) inbred mice were from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All mice used in these studies were bred and raised in our facility at the Wadsworth Center. Inbred and knockout strains (B6, 129P2, *Il18*^{-/-}, *Il18r1*^{-/-}) were maintained by brother-sister matings. The three F1 populations (B6/*Il18r1*^{-/-}F1, 129P2B6F1, 129P2/*Il18r1*^{-/-}F1) were generated in our facility. Mice were housed in same-sex groups of 2–4 per cage in clear Plexiglas cages (29 cm L, 18 cm W, 12.5 cm H) with stainless steel wire lids and filter tops, in a temperature-controlled (68–72°F) room, with food (LabDiet 5PO4 Autoclavable Prolab RMH 3500) and water available *ad libitum*. Mice were maintained on a 12 hr light/dark cycle (lights on at 7:00 AM). Behavior testing was conducted during the light phase of the light/dark cycle, using adult animals between 60 and 90 days of age. Only group-housed animals naïve to the test battery were used for behavioral testing. All procedures had prior approval by the Wadsworth Center Institutional Animal Care and Use Committee.

2.2 Exploratory Behavior

Exploratory behavior was quantitated using Versamax automated activity monitors (42 cm L, 42 cm W, 30 cm H; Accuscan Instruments, Columbus, OH) enclosed in melamine sound-attenuating chambers (65 cm L, 55 cm W, 55 cm H; Med Associates, St. Albans, VT). Mice were transported to the testing room 1 hr prior to the start of testing. Testing consisted of a 5-min session, repeated across 3 consecutive days or a 60-min session repeated across 2 consecutive days. Our 5-min/3-day procedure has been described in detail previously (Bolivar and Flaherty, 2003; Bolivar, 2009; Bolivar et al., 2000; Bolivar et al., 2001; Bolivar et al., 2002; Bolivar et al., 2003, 2004; Bothe et al., 2004, 2005; Cook et al., 2002) and will only be reviewed briefly here. Each mouse was weighed at the beginning of a session, placed in a clear Plexiglas holding cage for 5 min, and then placed in the center of the dark activity monitor for testing. The procedure for the 60 min test was identical, except for session length and number of days of repetition. The total distance traveled was recorded, as well as the percentage of time spent and percentage of total distance traveled in the center of the monitor. Intersession and intrasession habituation scores were also calculated (Nadel, 1968).

2.3 Elevated Zero Maze Assay

Anxiety-related behavior was quantitated using the elevated Zero Maze Digital Monitoring system (Accuscan Instruments, Columbus, OH). Details of the maze and procedure have been described elsewhere and will only be briefly reviewed here (Cook et al., 2002; Cook et al.,

2001). The maze consists of an elevated circular platform with two “open” and two “closed” quadrants. Closed quadrants consist of clear Plexiglas walls with an 8-photobeam configuration that detects locomotor activity. One hour prior to testing, the mice were placed in a darkened room (<1.0 lux). At the beginning of testing mice were individually removed from the home cage and placed at the threshold of a closed quadrant. The 5-min testing session began immediately upon the first beam-break, and total locomotor activity was recorded as photobeam breaks. Data were reported as percentage of time spent in the open quadrants, latency to first enter an open quadrant, and locomotor activity in the closed quadrants.

2.4 Modified Accelerating Rotorod Assay

We used the Smartrod Rotating Rod Apparatus (48 cm L, 11 cm W, 30 cm H; Accuscan Instruments, Inc., Columbus, OH) to assess motor coordination and a modified accelerating rotorod protocol. We have previously used this protocol to determine inbred strain differences in motor performance (Cook et al., 2002; McFadyen et al., 2003). Mice were transported to the testing room at least 1 hr prior to testing. A textured rod with a diameter of 3 cm was programmed to slowly accelerate from 1 to 15 rotations per min (rpm) within the first 60 sec, remaining constant at 15 rpm until deceleration occurred within the last 10 sec of each 3-min trial. Since weight has been shown to influence performance in the rotorod assay (Cook et al., 2002; McFadyen et al., 2003), each mouse was weighed immediately before testing. At the beginning of each trial, the test mouse was placed directly on the immobile rod, and the program was activated. The trial terminated either when the mouse fell, as detected by photobeams at the grid floor, or at the end of the min test. The mouse was given three trials, spaced at least 20 min apart, during the 1-day test protocol, to establish baseline motor performance. Latency to fall (active rotation, in sec) was recorded for each trial, and the data for the three trials were averaged. Animals that did not fall were assigned the maximum latency of 180 sec.

2.5 Genotyping of Flanking Region and Full Genome Scan

To determine the size of the 129-derived flanking region, we isolated and purified genomic DNA from tail biopsies, using the Puregene Core Kit (Qiagen Inc., Valencia, CA) according to manufacturer's specifications. DNA yield was quantitated by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). MIT microsatellite markers polymorphic between 129P2 and B6 were selected to define the 129P2-derived flanking region of *Il18r1*^{-/-} male mice. These markers were selected at intervals that provided informative coverage across Chromosome 1, particularly in the region containing the targeted *Il18r1* gene. Markers used in this study were D1Mit149, D1Mit103, D1Mit187, D1Mit135, D1Mit306, D1Mit134, D1Mit215, D1Mit19, D1Mit303, D1Mit214, D1Mit73, D1Mit478, D1Mit236, D1Mit477, D1Mit122.1, D1Mit232, D1Mit123, D1Mit318, D1Mit373, D1Mit211, D1Mit4, D1Mit67, D1Mit294, and D1Mit65.

Genomewide genotyping was conducted at Taconic Farms, Inc. (Rensselaer, NY) and used a 1449 single nucleotide polymorphism (SNP) panel (Medium Density Linkage Panel, Illumina, San Diego, CA) with independent samples from three *Il18r1*^{-/-} mice. For this analysis, 873 of these SNPs were informative between B6 and 129P2, and these SNPs were used to calculate the percentage of B6 genetic background.

2.6 Statistical Analyses

All experimental parameters were analyzed for significance by unpaired t-test or analysis of variance (one-, two- or three-way ANOVA). Significant effects from ANOVAs were then evaluated by Fisher's Protected Least Significant Difference (PLSD) post hoc tests. All statistical analyses were done with StatView 5.0 software (SAS Institute Inc., Cary, NC).

3. Results

3.1 Exploratory Behavior (5-min per day, for 3 days)

We measured the total distances traveled by *Il18r1*^{-/-}, *Il18*^{-/-}, and B6 male mice in the activity monitor across the 3 days of testing. Overall, *Il18r1*^{-/-} mice were less active than were either B6 or *Il18*^{-/-} mice (Figure 1). When the data were analyzed by mixed ANOVA, we found a main effect of strain ($F(2,120)=22.475$, $p<0.0001$), a main effect of day ($F(2,240)=99.540$, $p<0.0001$), and an interaction between strain and day ($F(4,240)=3.669$, $p=0.0064$). To evaluate this interaction in more detail, we calculated activity change scores (intersession habituation scores), which correct for baseline differences in activity levels (Nadel, 1968). These activity change scores were calculated as follows: Day 3 total distance/(Day 1 total distance + Day 3 total distance). When these scores were then analyzed by one-way ANOVA, no significant differences were seen among the strains ($B6=0.388\pm0.010$, *Il18*^{-/-} $=0.417\pm0.013$, *Il18r1*^{-/-} $=0.412\pm0.012$). Thus, it appears that baseline activity differences are primarily responsible for the significant differences seen across strains. Accordingly, first-day activity became the focus of our continued genetic studies.

3.2 Dissociation of *Il18r1* and 129P2-derived genetic contributions to behavior

Because both *Il18*^{-/-} and *Il18r1*^{-/-} strains have been made congenic on a B6 background, comparing each of the knockout strains to B6 is informative. On day 1 of testing, *Il18r1*^{-/-} mice traveled significantly less in the activity monitor than did B6 mice ($t(84)=5.560$, $p<0.0001$; Figure 2). In contrast, there was no significant difference between *Il18*^{-/-} knockout mice and B6 mice (Figure 2). Given that both *Il18r1*^{-/-} and *Il18*^{-/-} knockout/congenic mouse strains have been generated by conventional gene targeting (Hoshino et al., 1999; Takeda et al., 1998) using 129P2 embryonic stem cells, 129P2-derived genes could be the cause of the low activity behavioral phenotype seen in the *Il18r1*^{-/-} strain, rather than the targeted *Il18r1* gene. Accordingly, we also examined exploratory behavior in 129P2 mice. Inbred 129P2 mice, like *Il18r1*^{-/-} mice, traveled less during the 5-min session than did B6 mice ($t(55)=5.148$, $p<0.0001$; Figure 2). We next determined whether the behavior of the *Il18r1*^{-/-} mice is due to 129P2 genes rather than to the loss of the targeted *Il18r1* gene.

We dissociated the effects of the targeted *Il18r1* gene from the 129P2-derived genes, through a simple three-step breeding strategy (Bolivar et al., 2001). The first step of the strategy tests for a behavioral difference between the knockout strain and the B6 strain. We have established that *Il18r1*^{-/-} and B6 mice behave differently (Figure 2, Step 1). Having established this behavioral difference, we moved to Step 2 to determine the mode of genetic transmission for the causative gene(s). The distance traveled in the activity monitor by mice heterozygous for *Il18r1* and 129P2-derived genetic loci (B6*Il18r1*^{-/-}F1) was similar to the distance traveled by the B6 mice (Figure 2, Step 2), indicating that the behavioral deficit observed in the knockout mice is recessive. Behavioral comparison of 129P2B6F1 and 129P2*Il18r1*^{-/-}F1 mice (Figure 2, Step 3) showed significant differences in total distance traveled ($t(57)=2.509$, $p=0.015$). If ablation of the *Il18r1* gene was responsible for the recessive ambulation deficit, we would expect 129P2*Il18r1*^{-/-}F1 and 129P2B6F1 to display similar baseline activities because they are not homozygous for the mutant *Il18r1* allele. Therefore, it appears likely that 129P2 alleles for one or more genes are responsible for the activity deficit. 129P2*Il18r1*^{-/-}F1 mice, which carry two 129P2 alleles at specific genomic regions, display significantly lower ambulatory activity than do 129P2B6F1 mice, which possess only one copy of each 129P2 allele at any given locus.

3.3 Identification of 129P2-derived regions flanking the *Il18r1* gene and on other Chromosomes

To begin the process of identifying the gene or genes influencing the low activity phenotype in *Il18r1*^{-/-} mice, we next defined the limits of the 129P2-derived genetic material flanking the ablated *Il18r1* gene. We used MIT markers polymorphic between B6 and 129P2 to genotype the flanking region. Our results revealed several distinct regions of residual 129P2 material on Chromosome 1 (Figure 3). Consistent with our hypothesis, there was a region of 129P2 genes immediately proximal to the site of the ablated *Il18r1* gene, spanning approximately 10 Mb on Chromosome 1. In addition, we identified two other regions of residual 129P2 genetic material at approximately 60 and 100 Mb, on Chromosome 1.

We also considered that 129P2-derived material may reside elsewhere in the *Il18r1*^{-/-} genome, extending beyond the genomic region flanking the ablated *Il18r1* gene. Using informative SNPs between B6 and 129P2 in a high-throughput genomewide genotyping analysis, we confirmed our findings of 129P2 genetic material on Chromosome 1. In addition, we identified multiple genetic regions on other chromosomes that are either heterozygous for B6 and 129P2 alleles or homozygous for 129P2 alleles for each of the *Il18r1*^{-/-} animals genotyped in this analysis. Regions of the *Il18r1*^{-/-} genome that are homozygous for 129P2 alleles include: Chr 1 (40.2–69.4 Mb), Chr 4 (59.1–84.5 Mb and 107.0–128.0 Mb), Chr 5 (141.5–147.0 Mb), Chr 8 (67.2–95.3 Mb), Chr 10 (102.0–128.3 Mb), and Chr 14 (92.0–116.4 Mb).

3.4 Exploratory Behavior (60-min per day for 2 days)

Independent of the identity of the actual gene or genes responsible, it was also important to describe the low-activity phenotype in more detail. An understanding of the basis of the phenotype will help in identifying the gene or genes involved. Is the low activity of *Il18r1*^{-/-} in the open field unique to the short 5-min exposure testing time, or is it retained in a longer testing session? To examine the effects of a longer period of time for exploration, we tested mice for 60 min per day, on 2 consecutive days. This approach allows time for mice to recover from many acute stressors, such as handling, separation from cagemates, and abrupt exposure to novel or altered environmental factors (lighting, odors, sounds). We also included females in our investigations at this stage, to determine whether the low-activity phenotype applies to both sexes or is exclusive to males.

To examine total distance traveled in the open field over the 60-min period, we divided the session into 10-min blocks, and used a mixed ANOVA to analyze the data for each day separately. There was no main effect of sex, nor did sex interact with any other variable for any of the exploratory behavior analyses. Therefore, although both sexes are included in all figures, comparisons between strains were collapsed across sex. For day 1, ambulatory activity, we observed a main effect of strain ($F(2,108)=50.493$, $p<0.0001$), a main effect of block ($F(5,540)=331.788$, $p<0.0001$), and an interaction between strain and block ($F(10,540)=3.386$, $p=0.0003$) (Figure 4). To examine this interaction in more detail, we calculated intrasession habituation scores as follows: total distance traveled in the last 10-min block / (total distance traveled in the last 10 min block + total distance traveled in the first 10 min block). These activity change scores were then analyzed by two-way ANOVA. A main effect of strain was seen ($F(2,108)=6.577$, $p=0.002$; Table 1). Despite their overall lower activity level, *Il18r1*^{-/-} mice showed a greater drop in activity across the testing session (increased intrasession habituation) than did B6 mice ($p=0.0008$). On day 2, there were main effects of strain ($F(2,108)=26.678$, $p<0.0001$) and block ($F(5,540)=94.847$, $p<0.0001$), but no significant interaction between the two variables. Throughout both testing sessions, the total distance traveled for *Il18r1*^{-/-} mice was lower than the distances traveled by B6 and *Il18*^{-/-} mice (Figure 4).

As we had done for the shorter (5-min) test, we calculated intersession habituation scores for the 60-min test (Nadel, 1968) as follows: Day 2 total distance/(Day 1 total distance + Day 2 total distance). When these activity change scores were then analyzed by two-way ANOVA, no significant differences were seen among the strains or between the two sexes (B6 males=0.420±0.015, *Il18*^{-/-} males=0.424±0.013, *Il18r1*^{-/-} males =0.454±0.012, B6 females=0.437±0.015, *Il18*^{-/-} females=0.447±0.018, *Il18r1*^{-/-} females =0.421±0.021).

We considered that the lower activity level of *Il18r1*^{-/-} mice could be the result of heightened anxiety, thus we examined center-avoidance behavior in this assay and calculated the percentage of time spent in and total distance traveled in, the center of the monitor during the 60-min session on Day 1. There was a main effect of strain on time spent ($F(2,108)=23.427$, $p<0.0001$, Figure 5A) and distance traveled ($F(2,108)=24.384$, $p<0.0001$; Figure 5B) in the center of the monitor. Fisher's post hoc tests revealed that *Il18r1*^{-/-} mice had significantly lower ambulation in the center ($p<0.0001$) and spent significantly less time in the center ($p<0.0001$), relative to B6 mice.

3.5 Zero Maze Behavior

Center-avoidance behavior in the open field is suggestive of anxiety-like behavior (Crawley, 2007; Cryan and Holmes, 2005; Singer et al., 2005; Tang and Sanford, 2005). Therefore, we further assayed potential anxiety differences among *Il18r1*^{-/-}, *Il18*^{-/-}, and B6 mice, through evaluation of elevated zero maze activity. We used two-way ANOVAs with strain and sex as between-subjects variables to evaluate percentage of time in the open quadrants, latency to enter the open quadrants, and activity within the closed quadrants of the maze. Both sexes are included in Figure 6 for comparison purposes; however, post hoc strain comparisons were collapsed over sex as there was no interaction between sex and strain for any of the zero maze variables.

For the percentage of time spent in the open quadrants of the elevated zero maze, there was a main effect due to strain ($F(2,127)=20.939$, $p<0.0001$; Figure 6A) and a main effect of sex ($F(1,127)=8.777$, $p=0.0036$). *Il18r1*^{-/-} mice spent less time in the open quadrants of the zero maze than did B6 mice ($p=0.0008$). In contrast, *Il18*^{-/-} mice spent significantly more time in the open quadrants than did B6 mice ($p=0.0053$). Collapsed over strain, females spent more time in the open than did males ($p=0.0025$). We analyzed the time until the first entry into one of the open quadrants of the maze by 2-way ANOVA, with strain and sex as between-subjects variables. There was a significant main effect of strain ($F(2,127)=4.033$, $p=0.02$; Figure 6B), with *Il18r1*^{-/-} mice showing longer latencies than B6 mice ($p=0.0036$). There was no difference between B6 and *Il18*^{-/-} mice. There was no main effect of sex or interaction between sex and strain.

One complication in analyzing elevated zero maze data is that examination of the amount of time spent in the open quadrants, and latency to enter these quadrants does not take into account baseline activity differences across strains. The reason why an animal does not enter the open quadrants could be either high anxiety or low activity. The corrected activity calculation is an assessment of the activity in the closed quadrants per amount of time spent in those areas, and that calculation can be used to characterize general ambulatory activity in the maze. When we analyzed the scores by a two-way ANOVA with strain and sex as between-subjects variables, we obtained a main effect of strain ($F(2,127)=47.262$, $p<0.0001$; Figure 6C). The *Il18r1*^{-/-} mice were significantly less active in the closed quadrants than were B6 mice ($p<0.0001$), a finding that may in part explain the lower percentage of time spent by *Il18r1*^{-/-} mice in the open quadrants of the maze. In contrast, *Il18*^{-/-} mice do not exhibit higher activity than B6, either in the zero maze or the open field (Figures 1, 2, 4). Therefore, the greater amount of time spent by *Il18*^{-/-} mice in the open quadrants of the zero maze suggests that this strain is significantly less anxious in that environment than are their B6 counterparts.

3.6 Rotorod Performance

Impaired locomotor ability and coordination may negatively influence activity levels in the exploratory behavior task. To evaluate basic motor coordination, we examined rotorod performance in B6, *Il18*^{-/-}, and *Il18r1*^{-/-} strains. In terms of fall latency, i.e., the length of time for which the mouse remained on the rod before falling, we found main effects of strain ($F(2,114)=11.282$, $p<0.0001$) and sex ($F(1,114)=17.643$, $p<0.0001$), but no interaction between strain and sex (Figure 7A). Surprisingly, *Il18r1*^{-/-} mice remained on the rod significantly longer than did B6 mice ($p<0.0001$), indicating that the knockout mice were not deficient in general motor ability or coordination. Overall, females remained on the rod significantly longer than did males ($p<0.0001$), a finding consistent with our previous inbred strain study (McFadyen et al., 2003).

Body weight can influence performance in the rotorod assay (McFadyen et al., 2003), necessitating assessment of sex- and strain-specific differences in body weight. We found no main effect of strain (Figure 7B), therefore, the enhanced rotorod performance of *Il18r1*^{-/-} mice is not the result of lower body weight. There was a main effect of sex ($F(2,114)=290.598$, $p<0.0001$). Females of all three strains weighed less than did the respective males; the latter fact may explain, at least in part, the females' superior performance on the rotorod task (Figure 7B).

4. Discussion

Traditional gene-targeting technologies have provided a wide variety of knockout mouse strains, which can be studied to help us to elucidate how the immune system works and how it interacts with other body systems. However, some aspects of these technologies also provide the foundation for incorrect assumptions about the true role of a targeted gene. Even after a knockout strain has been made congenic with a B6 background, the flanking region surrounding the targeted gene contains 129 genes that, at least partially, could be responsible for the observed phenotype, given that most knockout strains are developed using embryonic stem cells from the 129 substrains. This caveat is an especially important consideration when the phenotype shows no apparent relationship to the target gene or its gene product. Although a number of reviews have described both the problems working with knockout strains and the possible solutions (Crusio, 2004; Eisener-Dorman et al., 2009; Gerlai, 1996; Wolfer et al., 2002), it is critical that a concrete example be provided for the above-described phenomenon. In an earlier study with the *Il10* knockout mouse, we found that a locomotor phenotype was due to material in the flanking region rather than the targeted gene (Rodrigues de Ledesma et al., 2006). The present study confirms that our finding for the *Il10* knockout was not an isolated case, and underscores the necessity that researchers remain aware of the problems associated with efforts to directly connect ablation of a gene with a phenotype observed in a knockout mouse model.

We examined behavior in two knockout strains modeling different means of disrupting the IL-18 pathway (*Il18*^{-/-} and *Il18r1*^{-/-}), to determine whether ablation of *Il18* or *Il18r1* genes would influence, either directly or indirectly, the CNS. We chose the above two strains for our investigations because IL-18 is produced by a variety of cells in the CNS (Cannella and Raine, 2004; Conti et al., 1999; Culhane et al., 1998; Sugama et al., 2002) and because behavioral abnormalities have recently been reported in mice lacking a functional *Il18* gene (Yaguchi et al., 2010). Thus, any behavior different from B6 could be due either to the ablated gene or residual 129P2-derived material.

4.1 *Il18r1*^{-/-} mice exhibit an ambulatory activity deficit due to 129P2-derived loci

We found a low exploratory behavior phenotype in the *Il18r1*^{-/-} knockout mouse strain. This phenotype was evident across multiple assays and experimental conditions and is not likely due to the absence of IL-18 signaling, in that IL-18 deficient mice did not display exploratory behavior abnormalities. Because there is no known relationship between the *Il18r1* gene and exploratory behavior, it was necessary to evaluate whether this phenotype was due to the targeted gene or to the presence of 129-derived genetic material. Our findings provide evidence that the low-activity phenotype is not due to the targeted *Il18r1* gene, but rather relates to the remaining 129P2-derived loci.

We also determined that the ambulatory activity trait exhibited by *Il18r1*^{-/-} mice is transmitted by a recessive mode of inheritance. 129P2 mice, but not B6 *Il18r1*^{-/-}F1 mice, exhibited ambulatory activity similar to *Il18r1*^{-/-} mice, indicating that this recessive trait is likely due to one or perhaps several 129P2-derived genes. This finding is not surprising, given the physiological and behavioral differences known to exist between B6 and 129 substrains (Simpson et al., 1997). In fact, comparisons of 129P2*Il18r1*^{-/-}F1 and 129P2B6F1 mice further support that the low activity is not due to the targeted gene. Our genomewide genotyping data clearly indicate that a substantial amount of 129P2-derived genetic material remains on Chromosome 1, as well as on several other chromosomes, despite the fact that this knockout strain has been extensively backcrossed to have a congenic B6 genetic background. Given the large number of genes located within these regions, further mapping is necessary to identify the specific gene(s) contributing to the ambulatory deficits in *Il18r1*^{-/-} and 129P2 mice.

Our identification of regions of homozygous 129P2-derived material in the *Il18r1*^{-/-} genome drastically reduces future mapping efforts, as our data suggest that these regions encompass the gene(s) responsible for the recessive exploratory activity trait. Several previously identified behavioral quantitative trait loci (QTL) within these 129P2 homozygous regions may contribute to this phenotype, including QTL for anxiety (Nakamura et al., 2003), circadian activity levels (Shimomura et al., 2001), cocaine-induced activation (Gill and Boyle, 2003), novelty/stress-induced locomotor activation (Gill and Boyle, 2005), and exploratory and excitability behaviors (Zhang et al., 2005). Similarly, QTL for olfactory bulb size (Williams et al., 2001), cerebellar cAMP levels (Kirstein et al., 2002), and cerebellum weight (Airey et al., 2001) may also play a role in the low exploratory activity observed in the *Il18r1*^{-/-} strain. Genes relevant to cardiovascular function may also affect mouse activity levels, and our 129P2-derived genetic regions contain QTL influencing atherosclerosis (Wang et al., 2007) and blood pressure (Nishihara et al., 2007; Sugiyama et al., 2001). Two QTL affecting visual function are also present within these regions (Danciger et al., 2003; Wolf et al., 2004). Thus, the QTL reviewed above are reasonable candidates for guiding further mapping studies.

All of the above evidence points to 129P2 genes, rather than the targeted *Il18r1* gene being responsible for the observed activity phenotype. Importantly, even mice that have been made congenic by extensive backcrossing to B6 retain small regions of 129-derived material in areas elsewhere in the genome that are resistant to genetic recombination. Thus, we must logically consider that one or more 129P2 genes in these regions may be responsible for a given phenotype, in addition to the 129P2 genes flanking the targeted gene. Our data supports these hypotheses, as pockets of 129P2-derived material have been retained in the B6 background of the *Il18r1*^{-/-} mouse, and this finding has been independently validated (Dirk Smith, Amgen, personal communication).

4.2 Anxiety-related behavior may influence the *Il18r1*^{-/-} ambulatory activity deficit

We hypothesized that the *Il18r1*^{-/-} ambulatory deficit was due to either physical (e.g., muscle weakness, excessive fatigue) or psychological (e.g., anxiety, stress, depressive-like behavior)

impairment. When evaluated in the open field assay, a common paradigm for assessing anxiety-related behavior (Crawley, 2007), *Il18r1*^{-/-} mice exhibited reduced ambulation and time spent in the center. If initial stress associated with the novel environment is causing or influencing the observed activity phenotype, *Il18r1*^{-/-} mice would be predicted to eventually recover from the stress effects, and exhibit an activity pattern more similar to those of B6 and *Il18*^{-/-} mice, by the end of the open field session. However, we found the *Il18r1*^{-/-} mice to be consistently less active for the duration of testing in the open field, even in lengthy (60-min) sessions. These results suggest that *Il18r1*^{-/-} ambulation is not decreased due to state anxiety or excitement, which are more transient responses to external stimuli (e.g., handling, sudden removal from their home cage, exposure to a novel environment) (Mill et al., 2002). Estrous cycle may also influence behavioral outcomes on some assays in some strains of mice (Meziane et al., 2007); however, similar phenotypic trends were observed for both males and females, suggesting that the *Il18r1*^{-/-} phenotype is not attributable to confounds related to the estrous cycle. It is possible, albeit unlikely, that the 60-min observation period still does not permit ample time for *Il18r1*^{-/-} mice to recover from the impact of such stressors. However, *Il18r1*^{-/-} mice may have an intrinsic higher basal anxiety level (trait anxiety) relative to B6 mice, and that could explain the persistence of the activity phenotype.

The activity pattern of *Il18r1*^{-/-} mice within a session (intrasession habituation) reveals an elevated capacity for short-term learning and memory or adaptation relative to B6 mice. This is particularly interesting in light of the low level of exploratory activity seen in the *Il18r1*^{-/-} strain. Enhanced habituation within a session generally implies that the test subject is able to quickly adapt to its novel surroundings. Given that stress- and anxiety-based responses often impair habituation, the fact that *Il18r1*^{-/-} mice display enhanced habituation relative to B6 mice suggests that the learning and memory capacity of these mice is not influenced by stress or anxiety, at least under baseline conditions.

To further investigate the possibility that an anxiety phenotype is influencing the observed activity phenotype, we tested the mice in the elevated zero maze. The *Il18r1*^{-/-} mice spent less time in the open quadrants and showed longer latencies to enter the open quadrants relative to B6, but their activity levels inside the closed sections of the maze were also reduced, relative to B6. Thus, it is difficult to make a final determination as to whether the reduced activity seen in this knockout strain is due to anxiety. The design of the elevated zero maze takes advantage of the instincts of rodents to explore novel surroundings, minimize their exposure in an unprotected environment, and avoid heights (Bouwknicht and Paylor, 2008; File et al., 2004). However, this anxiety paradigm does not measure the same type of anxiety-related behavior as does the open field assay; the latter induces moderate levels of anxiety due to the openness and novelty of the environment, coupled with the animal's inability to escape (Kalueff et al., 2007; Tang and Sanford, 2005). Anxiety in the elevated zero maze is defined in terms of activity in the open quadrants, but increased time in the open quadrants is non-specific: it can indicate that the mouse is hyperactive, less anxious, or highly determined to locate an escape route. Taken together, these anxiety-behavior measures suggest that *Il18r1*^{-/-} mice are more anxious than B6, although it is difficult to distinguish between high anxiety and low activity.

Interestingly, *Il18*^{-/-} mice display reduced anxiety relative to B6, which is in agreement with a recent behavioral evaluation of the strain (Yaguchi et al., 2010). Given findings implicating IL-18 in retinal vascularization in early postnatal stages (Qiao et al., 2007; Qiao et al., 2004), we have also considered that mice deficient in IL-18 may have impaired visual acuity that may affect their performance in behavioral tests. The fact that our exploratory activity data show that *Il18*^{-/-} mice perform similarly to B6 controls suggests that visual impairment is not an experimental confound for this phenotype. Perhaps the role of vision was decreased in the exploratory behavioral performance, as our mice were tested in dim lighting conditions.

Furthermore, Qiao et al (2004) report that vacularization abnormalities in mice lacking IL-18 are undetectable by P84; therefore, the adult knockout mice included in our study are unlikely to possess such abnormalities due to their age at the time of testing. This low-anxiety phenotype warrants further characterization, particularly since the functional contributions of the 129P2-derived flanking region and genetic background have not yet been addressed for the *Il18*^{-/-} strain.

4.3 Reduced locomotor capabilities are unlikely to account for the *Il18r1*^{-/-} ambulatory activity deficit

Alternatively, the consistently low activity levels exhibited by *Il18r1*^{-/-} mice could be indicative of reduced locomotor capabilities; if that is the case, the lower activity levels are ultimately due to either a physiological deficiency or else a reduced motivation to explore novelty as a consequence of sickness behavior. We found no evidence to support the idea that the *Il18r1*^{-/-} ambulatory deficits are due to poor muscle coordination or physical disability. Another potential explanation for the *Il18r1*^{-/-} activity deficit seen in both the open field and elevated zero maze is that *Il18r1*^{-/-} mice are not sufficiently motivated to perform in these assays. Both behavioral assays permit free exploration, thus, mice may explore either to gain information about their surroundings or to find an inconspicuous hiding place. The rotarod, however, forces the mice to either continue to move or to fall. Further studies are necessary to determine the role of motivation in performance on these assays.

4.4 Summary of *Il18r1*^{-/-} ambulatory activity deficit

The most significant finding of the present study is that the targeted gene is not always the source of the observed phenotype. It is important to remember that the ability to determine the specific role of the targeted gene can be hampered by the presence of pockets of residual 129 genomic DNA outside the region flanking the targeted mutation (Eisener-Dorman et al., 2009). Consequently, investigation of the putative phenotypic effects of non-B6 loci introgressed into the genetic background is essential. Although the confounding factor that is posed by genetic background may appear daunting to some researchers, it must be kept in mind when any knockout strain made by traditional methodologies is being evaluated. We have now documented two cases in which a behavioral phenotype is not due to the targeted gene. Our findings should serve as caveat to those who obtain phenotypes inconsistent with the known function of the targeted gene. Sometimes the complicated interpretation required to link a specific targeted gene to a given phenotype is not advisable, and Occam's razor is a preferable explanation: the effect is caused by residual 129 genetic material.

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Figure 1.

Exploratory behavior in the 5-min assay. Mean (+SEM) total distance traveled in the activity monitor over 3 consecutive days of testing. A total of 123 male mice were tested (N=48 B6, N=37 *Il18*^{-/-}, N=38 *Il18r1*^{-/-}).

Figure 2.

Schematic of approach used to separate effects of $Il18r1^{-/-}$ targeted gene from effects due to presence of other 129-derived genes in the flanking region (left panel), and application of the approach to the measurement of exploratory activity in the 5-min assay (right panel). Mean (+SEM) total distance traveled in the activity monitor during Day 1 of the 3-day assay. A total of 216 male mice were tested (N=48 B6, N=37 $Il18r1^{-/-}$, N=38 $Il18r1^{-/-}$, N=9 129P2, N=25 B6 $Il18r1^{-/-}$ F1, N=24 129P2B6F1, N=35 129P2 $Il18r1^{-/-}$ F1). * $p < 0.01$ vs B6, + $p < 0.05$ vs 129P2B6F1.

Figure 3.

The 129P2 gene region flanking the targeted *Il18r1* gene. Microsatellite markers mapped to Chromosome 1 revealed an approximately 30 Mb region of 129P2-derived genetic material remaining in the genetic background of *Il18r1*^{-/-} mice.

Figure 4.

Exploratory behavior in the 60-min assay. Mean (+SEM) total distance traveled over 2 consecutive days, 60 min per day (divided into six 10-min bins). A total of 114 mice were tested (females: N=15 B6, N=15 *IL18^{-/-}*, N=20 *IL18r1^{-/-}*; males: N=18 B6, N=19 *IL18^{-/-}*, N=27 *IL18r1^{-/-}*).

Figure 5.

Center exploratory behavior on Day 1 of the 60-min assay. Percentage of session time spent in (A), and percentage of total distance traveled in (B), the center of the activity monitor. A total of 114 mice were tested (females: N=15 B6, N=15 *Il18*^{-/-}, N=20 *Il18r1*^{-/-}; males: N=18 B6, N=19 *Il18*^{-/-}, N=27 *Il18r1*^{-/-}). * p<0.01 vs B6 (collapsed over sex).

Figure 6.

Assessment of behavior in the elevated zero maze. Percentage of time spent in the open quadrants (A), latency until first entry into either of the open quadrants of the maze (B), and activity within the closed quadrants (C). Testing was a single 5-min session. A total of 133 mice were tested (females: N=21 B6, N=18 *Il18*^{-/-}, N=21 *Il18r1*^{-/-}; males: N=20 B6, N=23 *Il18*^{-/-}, N=30 *Il18r1*^{-/-}). * p<0.01 vs B6 (collapsed over sex).

Figure 7.

Assessment of locomotor capacity and coordination on the rotorod. Latency to fall (averaged across three trials) (A) and body weight (B). Testing consisted of a single session encompassing three trials, spaced at least 20 min apart. A total of 120 mice were tested (females: N=12 B6, N=15 *Il18*^{-/-}, N=21 *Il18r1*^{-/-}; males: N=19 B6, N=23 *Il18*^{-/-}, N=30 *Il18r1*^{-/-}). * $p < 0.01$ vs B6 (collapsed over sex).

Table 1Mean ($\pm$ SEM) Activity Change Scores (Intrasession Habituation)

Strain	Females	Males
B6	0.255 $\pm$ 0.025	0.267 $\pm$ 0.022
II18 $^{-/-}$	0.243 $\pm$ 0.025	0.235 $\pm$ 0.021
II18r $^{-/-}$	0.173 $\pm$ 0.034	0.173 $\pm$ 0.023

Note. Activity change scores were used to measure habituation. These scores were calculated as follows: total distance traveled in the last 10-min block/(total distance traveled in the last 10 min block + total distance traveled in the first 10 min block). A total of 114 mice were tested (females: 15 B6, 15 II18 $^{-/-}$, & 20 II18r1 $^{-/-}$; males: 18 B6, 19 II18 $^{-/-}$, & 27 II18r1 $^{-/-}$).