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Importin α4 deficiency induces psychiatric disorder-related behavioral deficits and neuroinflammation in mice

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Importin α4, which is encoded by the *Kpna4* gene, is a well-characterized nuclear-cytoplasmic transport factor known to mediate transport of transcription factors including NF-κB. Here, we report that *Kpna4* knock-out (KO) mice exhibit psychiatric disorder-related behavioral abnormalities such as anxiety-related behaviors, decreased social interaction, and sensorimotor gating deficits. Contrary to a previous study predicting attenuated NF-κB activity as a result of *Kpna4* deficiency, we observed a significant increase in expression levels of NF-κB genes and proinflammatory cytokines such as *TNFα*, *Il-1β* or *Il-6* in the prefrontal cortex or basolateral amygdala of the KO mice. Moreover, examination of inflammatory responses in primary cells revealed that *Kpna4* deficient cells have an increased inflammatory response, which was rescued by addition of not only full length, but also a nuclear transport-deficient truncation mutant of importin α4, suggesting contribution of its non-transport functions. Furthermore, RNAseq of sorted adult microglia and astrocytes and subsequent transcription factor analysis suggested increases in polycomb repressor complex 2 (PRC2) activity in *Kpna4* KO cells. Taken together, importin α4 deficiency induces psychiatric disorder-related behavioral deficits in mice, along with an increased inflammatory response and possible alteration of PRC2 activity in glial cells.

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INTRODUCTION

Accumulating epidemiological evidence have identified a plethora of genetic and environmental risk factors that contribute to the pathogenesis of psychiatric disorders. Recently, members of the importin α (karyopherin α: KPNA) family such as KPNA1 (human importin α5), KPNA3 (human importin α4), and KPNA4 (human importin α3) have been identified as possible genetic risk factors to several different psychiatric disorders including schizophrenia, depression, and substance use disorders [1–6]. These three importin α subtypes are expressed in the central nervous system (CNS) of humans as well as mice [7], and constitutive depletion in mice has been associated to disorder-related behaviors: *Kpna1* deficiency to reduced anxiety and other psychiatric disorder-related behaviors [8–10], and *Kpna3* deficiency to deficits in reward-seeking behavior [11]. In particular, a postmortem study has implicated human importin α3 (KPNA4) [12] in the pathology of schizophrenia [6], where significantly decreased nuclear factor-kappa B (NF-κB) pathway signaling, decreased p65 protein levels/nuclear activation, and *KPNA4* downregulation was found in schizophrenia brains, suggesting that decreased KPNA4 results in deficient nuclear transport of p65 in schizophrenia patients. Moreover, in the same study, an allele in a *KPNA4* expression quantitative trait locus (eQTL) was associated to increased schizophrenia risk, decreased *KPNA4* expression, and decreased prepulse inhibition (PPI), suggesting that *KPNA4* depletion could have roles in the pathogenesis of schizophrenia. Despite such evidence, there has been little insight on the causal relationship between *Kpna4* deficiency in relation to psychiatric disorder-related behavior.

Importin αs are a structural and functional subcategory of the importin (karyopherin) superfamily which mediate signal-dependent protein transport across the nuclear envelope [13, 14]. Importin αs participate in nucleocytoplasmic transport by forming a trimeric complex with classical nuclear localization signal (cNLS) containing cargo proteins, as well as importin β1; another member of the importin superfamily which facilitates passage through the nuclear pore complex (NPC) [15]. Importin α subtypes show differential expression patterns in various tissues, as well as having distinct, yet "partially redundant" binding specificities [16], implying that differential importin α expression

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can regulate accessibility of nuclear proteins to the nucleus [16–18]. Additionally, recent accumulating evidence suggests that importin αs are involved in non-transport functions such as spindle assembly, nuclear envelope assembly, lamin formation, protein degradation, and chromatin alteration [15, 19–22], as well as neuron-specific functions such as axonal transport [23]. However, the physiological implications of such widespread functions are still under extensive examination.

Mouse importin α4 categorizes in the same α3 subfamily with the closely related subtype importin α3, which is encoded by the *Kpna3* gene and shares common characteristics including cargo specificity for proteins such as regulator of chromosome condensation 1 (RCC1), tumor suppressor p53, and methyl-CpG binding protein 2 (MeCP2) [16, 24]. In particular, the α3 subfamily has been well characterized in the tumor necrosis factor alpha (TNF-α) induced nuclear translocation of NF-κB subunits p65 (RelA) and p50 [25–28], the pathway which the previous study suggested to be downregulated in schizophrenia patients [6]. In relation, a recent study has reported that *Kpna4* deficiency hinders NF-κB nuclear translocation in lung cells and disrupts antiviral responses, resulting in higher influenza lethality in mice [29]. Although a constitutively *Kpna4* deficient mice line has been reported to show decreased pain responsiveness and impairment of c-fos nuclear import in sensory neurons [30], there has been little investigation into the effects of *Kpna4* deficiency in psychiatric disorder-related behaviors and regulation of neuroinflammation. Further examination of such psychiatric disorder-related behaviors in *Kpna4* deficient animals is necessary to elucidate the roles of the importin α4 in regulation of brain function and behavior.

In this study, we used a recently developed importin α4 (*Kpna4*) knockout (KO) mouse line which show no apparent deficits in gross morphology, but exhibit male subfertility and deficiencies in sperm morphology, motility, and acrosome reaction capacity [21]. Using this knockout line, we found that *Kpna4* deficiency in mice induces psychiatric disorder-related behaviors, increased neuroinflammation, enhanced inflammatory responses in primary cultured astrocytes, as well gene expression patterns suggestive of enhanced inflammatory responses and altered polycomb repressor complex 2 (PRC2) activity in sorted adult glial cells.

RESULTS

KO mice exhibit psychiatric disorder-related behaviors

To investigate the effects of constitutive *Kpna4* deficiency on behavior, we conducted a behavioral test battery consisting of an open field test (OFT), elevated plus maze (EPM), Y-maze, social interaction test, inhibitory avoidance (IA), and prepulse inhibition (PPI) tests to assess psychiatric disorder-associated behaviors.

Locomotor activity and anxiety-like behavior were assessed in an OFT, where no significant differences were observed in novelty-induced locomotion (first 5 min) between all genotypes (Fig. 1A). However, KO mice spent significantly shorter durations of time in the center of the open field (Fig. 1B), suggesting higher levels of anxiety-like behavior. There was no significant difference between genotype in general levels of locomotion over the entire 60 min trial (Fig. 1C and Fig. S1A). Furthermore, similar to the results in the OFT, KO mice showed a significantly shorter percentage of time spent in the open arms in the EPM test (Fig. 1D), suggesting higher levels of anxiety-like behavior in the KO mice.

We examined the social behaviors of KO mice in a reciprocal social interaction test [31], where the number and duration of contacts between the nose point of one mouse, with the nose point, body center, or tail base of the other mouse was quantified as a measure of social interaction (total interactions; sum of all 3 per mouse). Significant decreases in KO mice were observed in both duration (Fig. 1E) and counts of social interactions (Fig. 1F), along with several individual measures (Fig. S1B–G).

To assess if *Kpna4* deficiency results in memory deficits, we assessed short-term spatial memory and avoidance learning in KO mice. In the Y-maze test, we did not observe any significant alterations in spontaneous alternation between all genotypes (Fig. 1G). In the IA task [32], 2W-ANOVA analysis revealed significant effects of both *Kpna4* deficiency (Genotype) and test day (Trial), as well as a significant interaction (Fig. 1H; 2W-RM-ANOVA; main effect of genotype: $F(2, 25) = 25.41$, $p < 0.0001$; main effect of test day (Trial): $F(1, 25) = 229.1$, $p < 0.0001$; interaction: $F(2, 25) = 20.96$, $p < 0.0001$). Inter-day comparison of latencies showed that WT mice showed a significantly longer latency to enter the dark chamber on day 2. In contrast, heterozygote (Het, *Kpna4*$^{+/-}$) and KO mice showed significantly reduced latencies to enter the dark chamber compared with their WT littermates.

To test whether *Kpna4* deficiency results in sensorimotor gating deficits, we administered a PPI test against an acoustic startle stimulus. There was no significant difference in startle response to the 120 dB pulse alone (Fig. 1I). In contrast, levels of PPI were significantly altered as a result of genotype (Fig. 1J; 2W-RM-ANOVA; main effect of genotype: $F(2, 44) = 9.930$, $p = 0.0003$; main effect of prepulse strength (Trial): $F(3.015, 132.6) = 32.05$, $p < 0.0001$; interaction: $F(8, 176) = 1.682$, $p = 0.1056$), with KO mice exhibiting significantly lower levels of PPI compared to WT mice in all types of trials (prepluse strength 74, 78, 82, 86, 90 dB).

Examination of morphology and expression of importin α subtypes in the KO brain

Examination of gross morphology in the KO brain sections revealed no apparent defects (Fig. S2A), and past studies [7] as well as examination of single-cell RNAseq databases [33, 34] show that *Kpna4* expression is not region or cell type specific in the brain. Moreover, we examined if *Kpna4* deficiency results in complimentary upregulation of other importin α subtypes (*Kpna1*, *Kpna2*, *Kpna3*, *Kpna6*) in brain tissue. In the PFC, although qRT-PCR analysis showed slight but significant increase in mRNA levels of closed-related importin α3 (Fig. S2B), importin α3 protein levels were not significantly altered (Fig. S2C, D). In addition, there was no difference in expression of other subtypes between WT and KO mice in the hippocampus (Fig. S2E). Furthermore, immunohistochemical staining showed nuclear localization of importin α4 across several different areas in the brain (Fig. S3).

Increased proinflammatory reactions in brains of KO mice

As importin α4 (KPNA4) has been well characterized in the nuclear transport for NF-κB [25, 29], and its downregulation has been predicted to perturb NF-κB signaling in postmortem studies [6], we sought to examine if such perturbations occur in the brains of KO mice. Regional expression levels of NF-κB genes and proinflammatory cytokines were assessed, examining five regions (prefrontal cortex: PFC, nucleus accumbens: NAc, dorsal hippocampus: Hipp, basolateral amygdala: BLA, cerebellum: Cere) sampled from mice used in behavioral testing (Fig. 2A and Fig. S4). We first quantified regional *Kpna4* mRNA levels in various regions, where expression was detected in all five regions with modest variation (Fig. 2B). We next quantified mRNA expression of *Rela* and *Nfkb1* in the KO brain where, unexpectedly, we observed significant upregulation of *Rela* in the PFC, as well as significant upregulation of *Nfkb1* and increasing trends of *Rela* in the in the BLA (Fig. 2C, D).

We further examined the regional expression of three typical proinflammatory cytokines: *Tnf-a*, *Il-1b*, and *Il-6* expected to be downregulated as a result of decreased NF-κB nuclear retention. Similar to *Rela* and *Nfkb1*, significant upregulation of all three genes was observed in the BLA of KO mice, along with significantly upregulated *Tnf-a* in the PFC, and trend towards increase of all three in the PFC and Hipp (Fig. 2E–G). Measurement of systemic changes in immune-related signal proteins in the

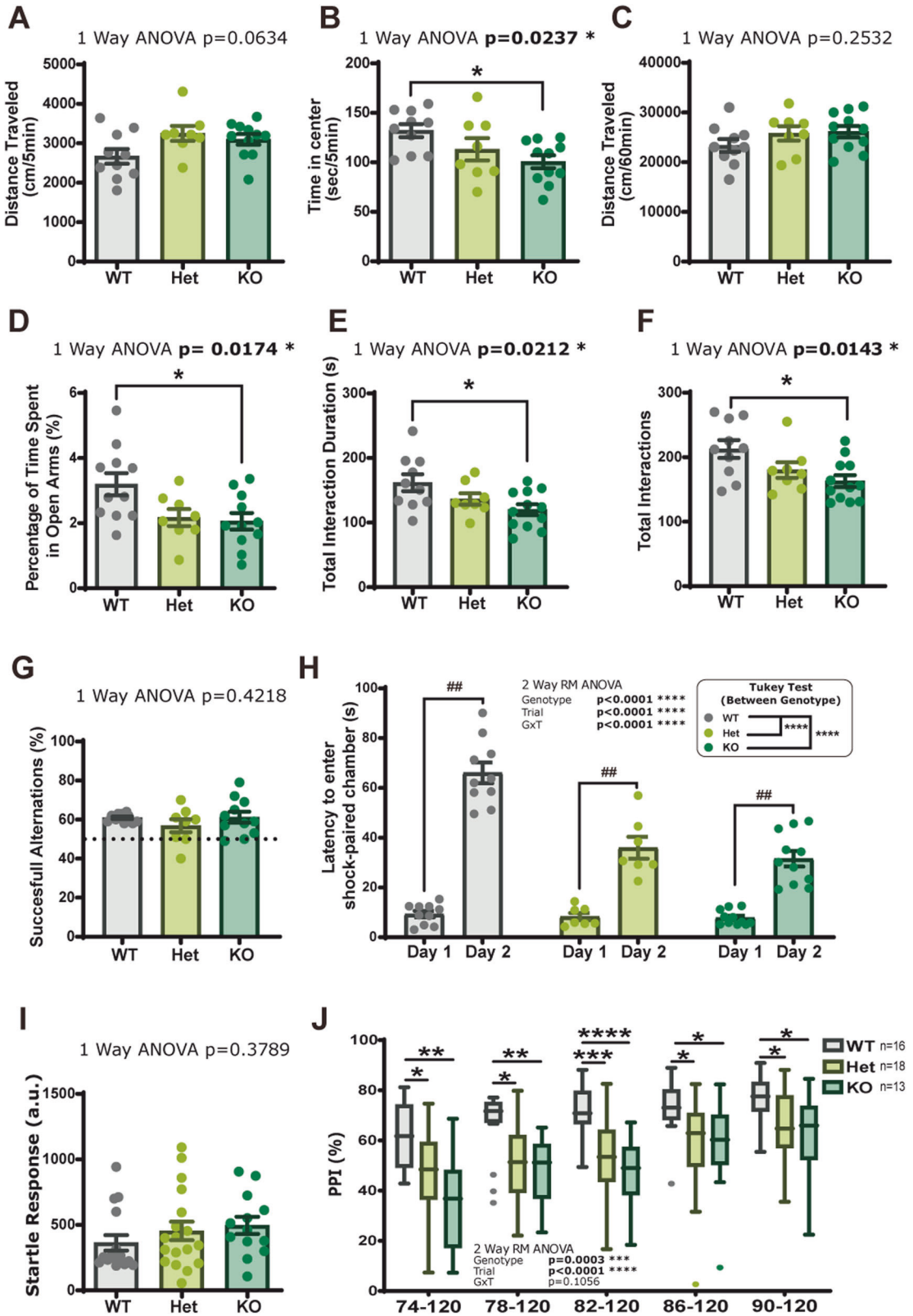


Fig. 1 Behavioral analysis reveals psychiatric disorder-related behavioral deficits in *Kpna4* deficient mice. A–C OFT. The total distance (**A**) and percentage of the time spent in the center (**B**) in the first 5 min of the OFT. Total distance (**C**) in the entire 60 min duration of the OFT. **D** EPM. The percentage of time spent in open arms during the EPM test. **E**, **F** Social interaction test. The duration (**E**) and number (**F**) of total interaction behaviors in the social interaction test. **G** Percentage of successful alternations in the Y-maze test. **H** IA. Latency to step through to the dark chamber in the IA test. **I**, **J** PPI. **I** Startle response when presented the only startle stimulus (120 dB). J Level of prepulse inhibition seen in the PPI test. Bar graphs represent mean ± SEM. Box and whisker plots represent median (center line), first and third quartiles (box), ±1.5 interquartile range (IQR) (whiskers), data points outside ±1.5 IQR are visualized as dots. Post-hoc Tukey's test between genotype: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Post-hoc Bonferroni test between days (**H**): ##$p < 0.01$. **A**–**H** WT 10–11, Het 7–8, and KO 11–12 **I**, **J** WT 16, Het 18, and KO 13.

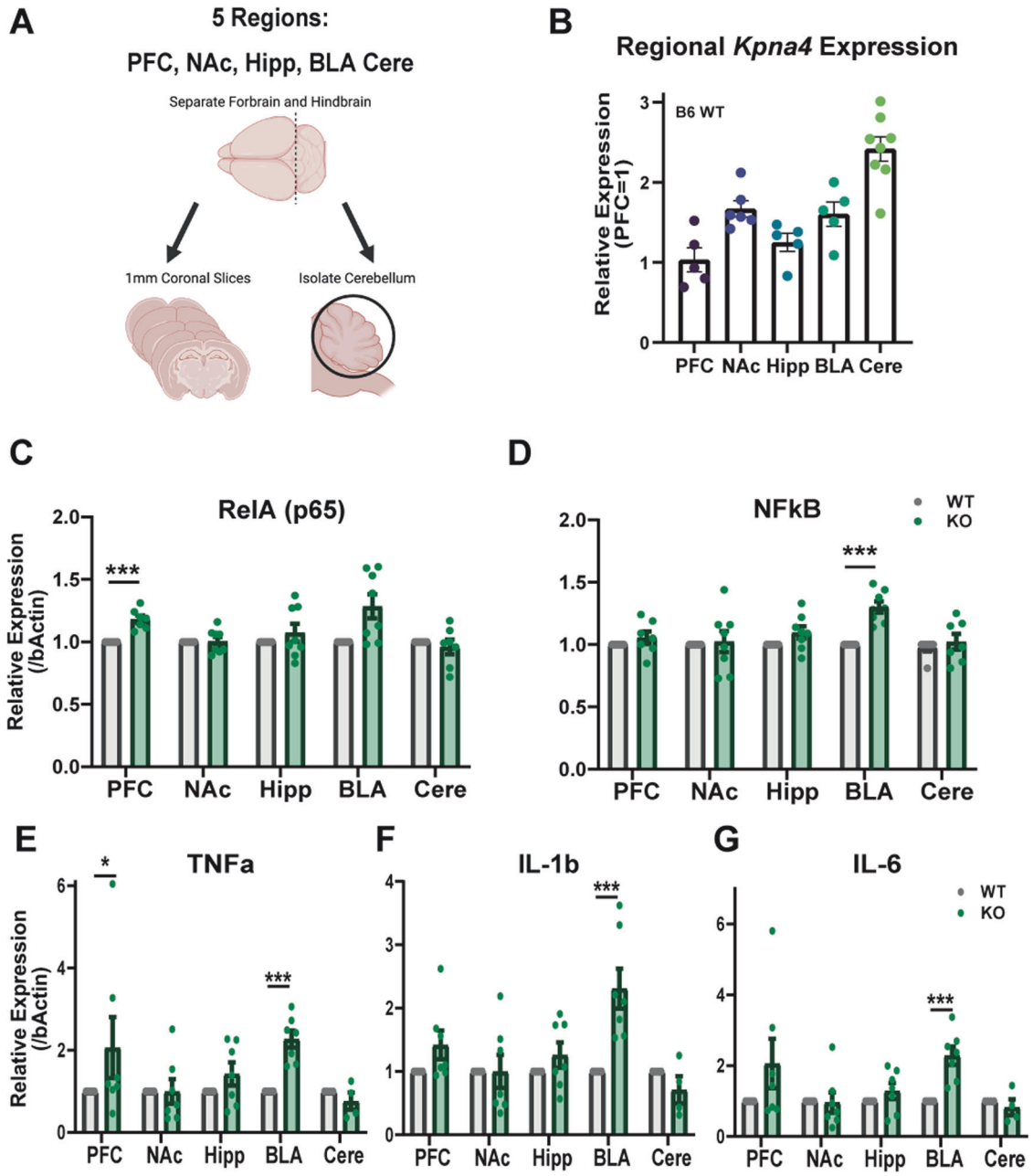
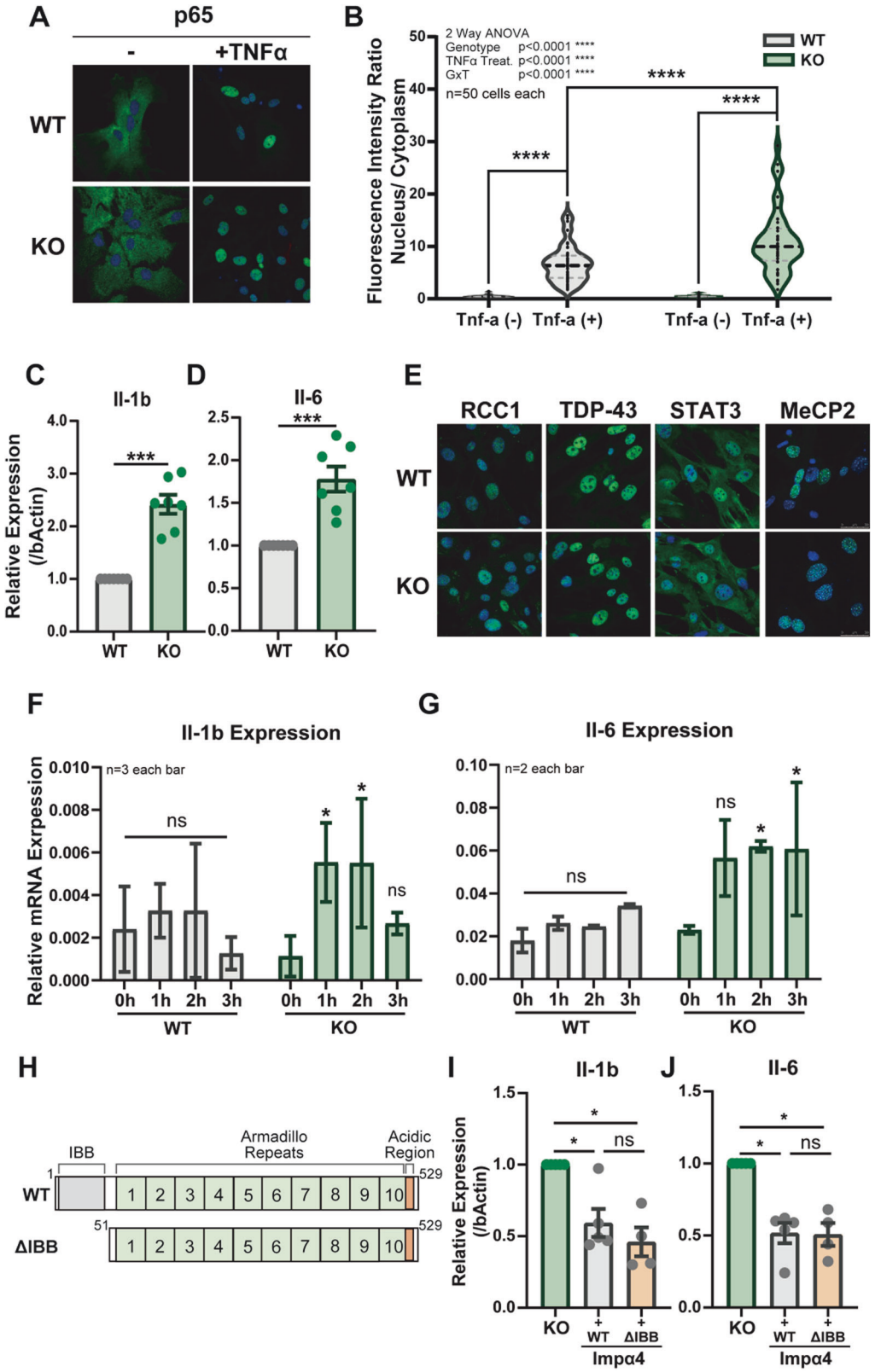


Fig. 2 Upregulation of NF-κB genes and proinflammatory cytokines in the KO mouse brain. A Scheme of regional sampling (created with BioRender.com.), coordinates shown in Fig. S3. **B** Regional expression levels of *Kpna4* (prefrontal cortex: PFC, $n = 5$; nucleus accumbens: NAc, $n = 6$; hippocampus: Hipp, $n = 5$; basolateral amygdala: BLA, $n = 5$; and cerebellum: Cere, $n = 8$). Regional mRNA expression levels (/β-actin) of **C** *Rela* and **D** *Nfkb1* in KO and WT mice (PFC, Hipp, BLA $n = 5$. NAc $n = 6$. Cere n = 4 each). Regional mRNA expression levels of **E** *Tnf-α*, **F** *Il1-β*, and **G** *Il-6* in KO and WT mouse brains (PFC, NAc, Hippo, BLA $n = 6$. Cere $n = 4$ each). Bar graphs represent mean ± SEM. **C**–**G** Mann–Whitney test: ***$p < 0.001$, and *$p < 0.05$.

same mice did not show any significant alterations (Fig. S5). Taken together, these data suggest that KO mice have increased proinflammatory responses associated with enhanced NF-κB signaling in the brain.

Increased proinflammatory activation in *Kpna4* deficient cells

As we observed unexpected increases in NF-κB genes and proinflammatory cytokines from the KO brain, we further investigated the effects of *Kpna4* deficiency on cellular inflammatory responses using primary cultured neural cells, focusing on glial populations. We first assessed TNF-α-induced nuclear translocation of NF-κB subunit p65 in primary astrocytes (AST). Clear nuclear translocation of endogenous p65 in response to TNF-α treatment was visible in both WT and KO cells in immunofluorescence experiments, revealing that *Kpna4* depletion does not alter their localization to the nucleus (Fig. 3A). Notably, the nuclear localization ratio of p65 in KO cells were significantly higher than WT (Fig. 3B). Moreover, examination of proinflammatory cytokine expression revealed that *Il-1b* and *Il-6* were upregulated in KO primary AST after TNF-α stimulation (Fig. 3C, D). Taken together with the results from KO brains, our observations suggest that *Kpna4* depletion results in an increase in inflammatory activation by increasing the concentration of p65 in the nucleus, rather than inhibiting nuclear transport of p65.



Furthermore, in our examination of mouse embryonic fibroblasts (MEFs) established from WT and KO mice, nuclear translocation of the other typical cargos specific for the importin α3 family such as RCC1 [35, 36], TDP-43 [37], STAT3 [38], and MeCP2 [24] were maintained in KO MEFs, indicating that *Kpna4* deficiency does not disturb their nuclear transport (Fig. 3E). Similar to our results in primary AST cells, time-course monitoring of *Il-1b* and *Il-6* expression after TNF-α stimulation showed that KO MEFs show increased inflammatory activation (Fig. 3F, G).

Fig. 3 Importin α4 depletion increases proinflammatory responses in cell culture and is suppressed by reintroduction of WT or transport-deficient (ΔIBB) KPNA4. A Immunofluorescence analysis of p65 (RELA) in *Kpna4*$^{-/-}$ astrocytes. Nuclear translocation of p65 in response to 30 min TNF-α stimulation was observed in the primary astrocytes established from KO and WT mice. **B** Fluorescence intensity ratio of nuclear vs cytoplasmic p65 in (**A**) ($n = 50$ cells each). mRNA expression levels of **C** *Il1-β* and **D** *Il-6* (/β-Actin) in KO and WT primary astrocytes after 3 h stimulation with TNF-α ($n = 7$ each). **E** Immunofluorescence analysis of RCC1, TDP-43, STAT3, MeCP2 in (unstimulated) KO and WT MEFs. Time-course changes of **F** *Il1-β* ($n = 3$ each bar) and **G** *Il-6* ($n = 2$ each bar) mRNA expression in KO and WT astrocytes. **H** Schematic representation of importin α4 (KPNA4)-WT and ΔIBB mutant transfected to KO cells. Expression levels of **I** *Il1-β* and **J** *Il-6* (/β-actin) in KO MEFs transfected with EGFP-importin α4 (Impα4)-WT, EGFP-Impα4-ΔIBB, or EGFP control after 1 h stimulation with TNF-α (KO $n = 5$, Impα4-WT $n = 5$, Impα4-ΔIBB $n = 4$). Bar graphs represent mean ± SEM. Violin plots represent median (solid line), first and third quartiles (dotted line). **B** Post-hoc Sidak's test: ****$P < 0.0001$. **C**, **D** Mann–Whitney test: ***$P < 0.001$. **F**, **G** Dunnett's test (vs 0 h) *$P < 0.05$. **I**, **J** Dunn's test *$P < 0.05$.

Results from our previous study have implied epigenetic alteration in the testis of *Kpna4* KO mice, which leads to altered gene expression, abnormal sperm formation, and infertility [21]. To address whether such epigenetic functions of importin α4 are involved in aberrant cytokine expression, we examined if KO MEFs can be rescued with importin α4 ΔIBB (IBB domain truncated, transport deficient) mutant as well as importin α4 WT (Fig. 3H). Both EGFP-Impα4-WT and EGFP-Impα4-ΔIBB transfected cells showed significantly lower *Il-1b* and *Il-6* expression after TNF-α stimulation compared to EGFP transfected controls (Fig. 3I, J). This result suggests that the effects of importin α4 in suppressing aberrant proinflammatory activation is dependent on non-transport functions such as chromatin alteration and epigenetic regulation, rather than its well-characterized transport functions.

Increased proinflammatory signaling and altered polycomb repressive complex activity in KO glial cells

To investigate the molecular alterations behind the unexpected increase in proinflammatory activation induced by *Kpna4* deficiency, we examined individual inflammation-related glial cell types to understand gene expression profiles in response to a proinflammatory stimulus. We applied a brain disassociation-cell sorting strategy utilizing magnetic cell sorting (MACS) or florescence-activated cell sorting (FACS), to isolate both microglial (MG) and AST populations from mice after LPS administration to stimulate inflammation (Fig. 4A and Fig. S6). In line with our previous tissue and cell experiments, we saw significant upregulation of *Il-6* expression in MG and AST isolated from adult KO mice using MACS, along with an increasing trend in *Tnf-a* and *Il-1b* (Fig. S7). Thus, we proceeded to isolate these populations with FACS to examine their gene expression profiles using RNAseq. In contrast with MG and AST, similar assessment of *Tnf-a*, *Il-1b*, and *Il-6* in non-neural immune cells (peritoneal macrophages: PECs) collected from the same mice showed no significant difference in expression of typical proinflammatory cytokines after LPS stimulation (Fig. S8).

Prominent perturbation of gene expression was observed in MG compared to AST (Tables S1 and S2), likely due to higher expression of LPS recognizing toll-like receptors in MG [33, 34]. Differential expression analysis identified 48 differentially expressed genes (upreg: 3, downreg: 45) of padj ≤ 0.05, |Fold Change| ≥ 2 in *Kpna4* deficient MG (Table S3). Notably, a larger number of downregulated genes compared to upregulated genes were identified, suggesting general repression of gene expression in both MG (Fig. 4B and Tables S1, S3) and AST (Fig. S9A and Tables S2, S4). As depletion of nuclear transport factors likely disrupts nuclear localization of specific transcription factors (TFs) or alter chromatin states [21], we sought to identify TFs and histone modifications upstream of altered genes by enrichment analysis in Enrichr [39–41]. By individually analyzing upregulated and downregulated gene sets of $p \leq 0.05$, |Fold Change| ≥ 2 (Tables S5–S14), we found significant enrichment only in the MG downregulated gene set (792 genes) (Fig. 4C), where significant enrichment of polycomb repressor complex 2 (PRC2) component TFs (SUZ12, EZH2, and JARID2) (Fig. 4C left and Table S8), as well as repressive histone modifications introduced by PRC2 (Fig. 4C right and Table S10) was seen, suggesting aberrant PRC2 activation and global downregulation of PRC2 target genes. To further validate these results, we utilized another analysis method: weighted parametric gene set analysis (wPGSA) [42], which allows for gene set-independent prediction of altered TFs directly from gene expression data. Application of wPGSA to MG data revealed prominent and significant enrichment of binding sites of PRC2-related TFs (EZH2, SUZ12, JARID2, EZH1, PHF19, RING2, etc.) in genes downregulated by *Kpna4* deficiency (t score ≤ 0), as well as suggesting a global downregulation of gene expression (i.e. the majority of TFs were t score ≤ 0) (Fig. 4D and Table S15). Moreover, the majority of TFs enriched in MG, including PRC2 component TFs EZH2 and SUZ12, were common between MG (Table S15) and AST (Table S16) wPGSA results (Fig. 4E and Fig. S9B), suggesting that increased PRC2 activity may underlie the increased inflammatory responses observed in KPNA4 deficient cells.

Finally, we performed cross-validation for our results with a previously reported dataset from *Kpna4* deficient neural cells (dorsal root ganglion, Marvaldi et al. [30]), and found similar enrichment of PRC2 component TFs in genes downregulated by *Kpna4* deficiency following tissue damage (day 7; Fig. S9E, F and Table S18). This further supports that *Kpna4* deficiency increases PRC2 activity and globally suppresses gene expression.

DISCUSSION

In this study, we demonstrated for the first time that an importin α4 (*Kpna4*) deficient mouse line exhibits increased anxiety-related behaviors, decreased social interaction, decreased avoidance learning, and decreased prepulse inhibition. As the previous study associating *KPNA4* to schizophrenia in postmortem samples had suggested contributions of *KPNA4* deficiency in the downregulation of NF-κB pathways in patients [6], we assessed the expression of NF-κB genes as well as downstream proinflammatory cytokines in KO mice. Contrary to initial predictions based on the study [6], we found that *Kpna4* deficiency did not decrease NF-κB nuclear localization, but instead causes an increase, along with increasing proinflammatory responses in KO tissues and cells. Moreover, this proinflammatory increase was rescued by addition of not only full length, but also transport-deficient (ΔIBB) mutants. We have previously demonstrated that this ΔIBB mutant of importin α can migrate to the nucleus by independently of importin β1 and Ran [43]. In addition, our immunohistochemical analysis demonstrated that importin α4 was observed in the nucleus of mouse brain tissue, suggesting that disruption of chromatin regulatory functions, not nuclear transport dysfunction, from importin α4 deficiency induces increased proinflammatory responses in correlation to abnormal behavior in mice. Finally, we examined gene expression in sorted adult glial cells from KO mice, finding evidence supportive of increased PRC2 activity underlying the perturbations in gene expression due to *Kpna4* deficiency.

In this study, we found altered behavioral characteristics in KO mice in anxiety-like behavior (OFT, EPM), social interaction, passive avoidance (IA), and PPI. This is in line with the initial report in Roussos et al. [6] that *KPNA4* deficiency associates to higher risks of schizophrenia and decreased PPI. Two previous studies [8, 30]

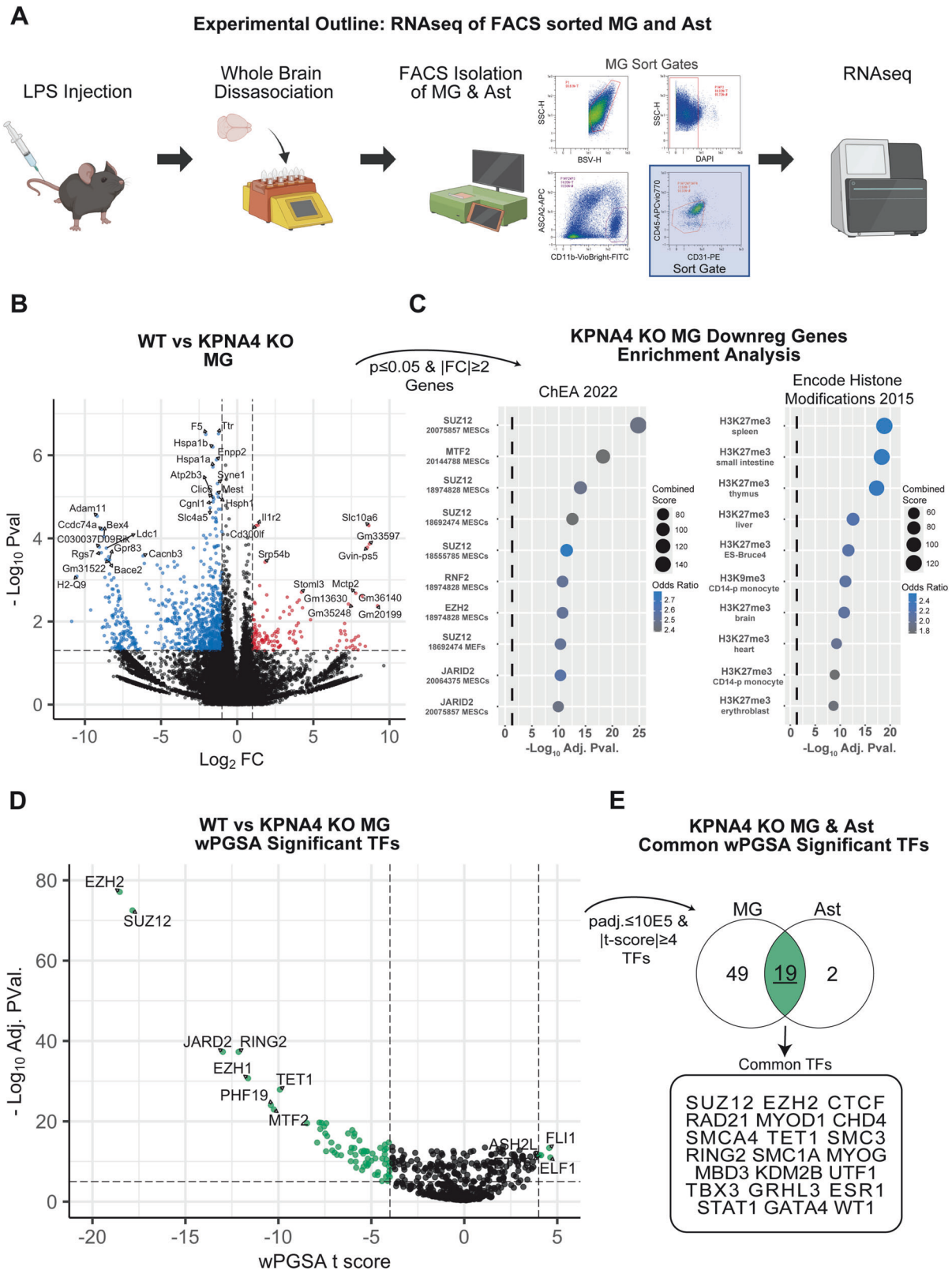


Fig. 4 RNAseq analysis of *Kpna4*$^{-/-}$ microglia reveal alterations genes regulated by polycomb repressor complex. A Outline of FACS experiments from KO and WT mice (created with BioRender.com.). **B** Volcano plot of genes with altered expression in KO MGs in comparison to WT. Horizontal axis: $\log_2$ fold change, vertical axis: $-\log_{10}$ *p* value, colored points: genes with $p \leq 0.05$ and |Fold Change| ≥ 2. **C** Enrichment analysis on MG downregulated gene list. Dotted line: adj.$P = 0.05$. **D** Volcano plot of wPGSA on MG data. Each point indicates each TF, horizontal axis: wPGSA t score, vertical axis: $\log_{10}$ P.Val. (BH adjusted). Colored points: TFs with padj ≤ 10E − 5 and |tscore| ≥ 4. **E** Venn diagram indicating TFs with significant enrichment in wPGSA in MG and AST.

have performed behavioral testing on *Kpna4* deficient mice. In particular, Panayotis et al. [8] have examined three behaviors (locomotion, anxiety-related behavior, and acoustic startle response), and have found alterations in home cage activity (higher and lower in KO than in WT in dark and light phases, respectively), no alteration in anxiety-like behavior, and increased startle response. Moreover, Malvaldi et al. [30] found decreased pain responses in KO mice. The discrepancy between our results and the past studies may be due to differences in experimental protocol, where the past studies have performed behavioral analysis during the "dark" phase of the light cycle, as well as differences in background substrains of the KO mice used (C57BL/6JJcl vs C57BL/6OlaHsd). Notably, as KO mice have been reported to exhibit decreased pain responses, the decreased latency to enter the shock paired chamber observed in our IA experiments may be due to attenuated pain response instead of deficits in avoidance learning.

From examination of schizophrenia postmortem patients, Roussos et al. [6] found NF-κB downregulation and significant association of KPNA4 genetic elements in the patient group. This lead to the prediction that *KPNA4* deficiency results in depletion of NF-κB factors from the nucleus, along with downregulation of NF-κB downstream genes including proinflammatory cytokines. This is contrastive to the inflammation hypothesis of schizophrenia, arising from observations that patients show neural and/or systemic proinflammatory upregulation, especially during acute phases [44–48]. In respect to this, the authors predicted that the downregulated NF-κB signaling in postmortem brains (chronic patients) may have resulted from compensatory NF-κB pathway suppression after initial activation during acute phases [6]. In our examination of NF-κB and proinflammatory cytokine expression in relatively young *Kpna4* deficient mice (8–10 weeks), we observed an increase in *RelA* mRNA expression, as well as repeated evidence of increased expression of proinflammatory cytokines (after stimulation) in *Kpna4* deficient cells collected from embryonic or relatively young mice (10 weeks). These observations support the prediction that *KPNA4* deficiency increases NF-κB signaling in younger patients during acute phase schizophrenia, in line with the inflammation hypothesis. Examination of NF-κB signaling during senescence in aged *Kpna4* deficient mice may provide insight on such age- and phase-related discrepancies.

Notably, in contrast to the latest meta-analysis supporting systemic inflammatory activation in both acute and chronic stages of schizophrenia [44], we did not observe signs of systemic inflammation from our cytokine measurements (Fig. S5). Thus, the observed effects of *Kpna4* deficiency on inflammatory increasing are likely cell type specific. Varying nuclear transport pathways and/or epigenetic states may produce different outcomes even when similar pathways (e.g. NF-κB, PRC2) are affected, as seen in Thiele et al. [29], where *Kpna4* deficient lung cells exhibited NF-κB downregulation and hindered antiviral response. As KPNA4 deficiency-induced proinflammatory activation seems to be mostly brain-specific, additional risk factors (e.g. increased lymphocytic activity [44]) are likely present in schizophrenic patients, which enable aberrant propagation of neuroinflammatory episodes to the rest of the body, resulting in systemic inflammation.

Considering that *Kpna4* deficiency alone did not result in exclusion of p65 from the nucleus in mice, contribution from redundant nuclear import pathways may sustain the import of nuclear proteins necessary for essential cellular functions such as inflammation. There is extensive evidence demonstrating redundancy in both classical [25, 26, 49, 50] and non-classical [50] nuclear import of NF-κB proteins, and depletion of *Kpna4* may not be sufficient to hinder import of a specific nuclear protein. Near-complete exclusion of essential TFs from the nucleus seem to be lethal, as we previously demonstrated that mice deficient in both *Kpna4* and *Kpna3* (known as major transporters of NF-κB [25]) were not viable [21]. Moreover, as transfection with a nuclear transport-deficient mutant (ΔIBB) was sufficient for reversing the proinflammatory increase, such increases may be a result of direct gene regulatory functions, rather than the nuclear transport functions, of importin αs.

In our analysis of gene expression perturbations in *Kpna4* deficient MG and Ast cells, we found prominent enrichment of binding sites for PRC2 components (EZH2, SUZ12, JARID2, etc.) in genes downregulated in KO. PRC2 is a chromatin modifying complex mainly known for repressive H3K27 methylation and has a plethora of roles in cellular functions such as neural differentiation, immunoinflammatory regulation, and tumor regulation [51]. Its enrichment in downregulated genes suggest that *Kpna4* deficiency results in increased PRC2 activity. KPNA4 has been identified as a binding partner for PRC2 component EZH2 [52], and its cellular depletion may alter EZH2 functionality to increase repressive activity of PRC2. Moreover, we revealed that rescue of the ΔIBB mutant was sufficient in reversing increases in proinflammatory responses, which implies that the immunosuppressive effects of importin α4 are mediated through direct gene regulatory functions, rather than transport. This is in agreement with our proteomics analysis in a previous study, which implied reduction of active chromatin states due to *Kpna4* deficiency. This reduction can be explained by increased PRC2 activity causing aberrant silencing of widespread loci [21]. In regard to increased immunoinflammatory activation in KO glial cells, we observed enrichment of TFs responsible for immunoinflammatory activation (FLI1, ELF1, ASH2L, ETV6) upstream of genes upregulated in KO cells. Increased PRC2 activity has been found to alter microglial polarization towards a proinflammatory (M1-like) status and induces upregulation of proinflammatory cytokines [53], which have been observed in our KO cells. The precise molecular interactions that mediate PRC2 hyperactivity in *Kpna4* deficient cells, and the implications in behavioral regulation is still unknown and calls for further detailed examination. However, there is accumulating evidence that increased immunoinflammatory activity [46, 54–56], as well as repression of gene expression (decreased gene expression [57], increase in histone deacetylase and/or repressive histone modifications [58–60]) are involved in the pathology of schizophrenia and/or schizophrenia-associated behaviors, and *Kpna4* KO mice may provide a useful tool to understand the molecular basis of behavioral dysregulation triggered by neuroinflammation.

A previous study has demonstrated significant enrichment of AP1 family factors in genes downregulated by *Kpna4* deficiency, as well as decreased nuclear localization of c-fos in *Kpna4* deficient peripheral sensory neurons [30]. However, we were not able to observe enrichment of c-fos or other AP1 factors in our analysis of expression patterns in FACS sorted adult glial cells. This may suggest that specific regulatory roles of importin α4 exist in various neural cell types, which are defined by varying expression patterns of different nuclear transport factors and cargo. Further studies are required for understanding the correlation between the expression and function of importin α4 in different cells and tissues.

Our results emphasize the roles of importin α4 in behavioral and neuroinflammatory regulation in relation to psychiatric disorders. Furthermore, as two other importin α subtypes (*Kpna1*[1,2] and *Kpna3*[3–5]) have been associated to psychiatric disorders, further understanding and comparison of subtype specific cargo binding capabilities and non-transport functions may be important in uncovering the molecular pathology of psychiatric disorders. Additionally, as exportin 7, an importin β family nuclear transport factor, is coded in one of the top schizophrenia-associated loci [61], further insight of relationships between nucleocytoplasmic transport and behavior is necessary to undercover the functions of nuclear import factors in the pathology of psychiatric disorders.

MATERIALS AND METHODS

Animals

Heterozygous (Het), and homozygous (KO) importin α4 (*Kpna4*) knockout, as well as wild type (WT) mice on a C57BL6/JJcl (CLEA Japan Inc., Tokyo, Japan) background were generated by mating male and female Kpna4 Het mice [21]. All animal experiments complied with institutional guidelines by the Institutional Safety Committee on Recombinant DNA Experiments (Nos. 04219 and 04884) at Osaka University, (No. 110083) and (No. DNA-420) at NIBIOHN, Animal Experimental Committee of the Institute for Protein Research at Osaka University (Nos. 29-02-1 and R04-01-1), the Animal Care and Use Committee of Kyoto University (No. MedKyo17071), and animal research committees of NIBIOHN (No. DS26-34).

Behavioral tests

The behavioral tests were administered to two different cohorts. Cohort 1 was administered the open field test (OFT), elevated plus maze (EPM), Y-maze, social interaction test, inhibitory avoidance (IA), in the above order. Cohort 2 was administered the prepulse inhibition (PPI) test and used for tissue collection. Behavioral tests were administered following previously reported general procedures with minor modifications [9, 10, 31, 32, 62].

Detailed protocols on behavioral testing, along with dissection, qRT-PCR, immunoblotting, cell culture, and RNAseq analysis are described in the Supplementary Materials. Detailed statistics including ANOVA details are available in the Supplemental Statistics file.

DATA AVAILABILITY

Any datasets and code which are a part of this study are available from the corresponding author upon reasonable request. Files from our RNAseq experiments have been reposited to Gene Expression Omnibus (GEO) as GSE264180.

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AUTHOR CONTRIBUTIONS

MOka, TH, and YM conceived the study. KS, MMorita, YA, MOka, TH, and YM designed experiments. KS, MMorita, MOtani, RO, KL, and YM performed data acquisition and analysis. KS, YA, TH, and YM interpreted data. KS and MMorita performed behavioral analysis. KS and YA performed RNAseq and wPGSA analysis. KS and MMatsumoto performed MACS and FACS isolation of glial cells. EK and AS contributed to multiplex immunoarray. TM, AS, KL, MY, YY, MOka, TH, and YM supervised the study. KS, MOka, TH, and YM wrote the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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COMPETING INTERESTS

MMatsumoto is a employee of Miltenyi Biotec K.K. (Tokyo, Japan). All other authors declare that no conflict of interest exists.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments complied with institutional guidelines by the Institutional Safety Committee on Recombinant DNA Experiments (Nos. 04219 and 04884) at Osaka University (No. 110083) and (No. DNA-420) at NIBIOHN, Animal Experimental Committee of the Institute for Protein Research at Osaka University (Nos. 29-02-1 and R04-01-1), the Animal Care and Use Committee of Kyoto University (No. MedKyo17071), and animal research committees of NIBIOHN (No. DS26-34). All animal experiments were conducted in compliance with the ARRIVE guidelines. No human subjects and materials were involved in this study.

ADDITIONAL INFORMATION

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