Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

The insertion of Neomycin cassette impairs maternal and social behaviors in Arc/Arg3.1 knock-out mice

Lucie Pellissier

lucie.pellissier@inrae.fr

Physiologie de la Reproduction et des Comportements https://orcid.org/0000-0001-7085-3242

Ana Dudas

Physiologie de la Reproduction et des Comportements (PRC) https://orcid.org/0000-0002-8189-3466

Emilia CAIRE

Abdurahman HASSAN A KUKU

Océane MARCHAND

https://orcid.org/0009-0008-2402-1662

Nicolas AZZOPARDI

Heba ELSEEDY

Vinesh JUGNARAIN

Hélène VACHER

Benoît Piégu

Physiologie de la Reproduction et des Comportements (PRC) https://orcid.org/0000-0003-4384-1726

Anil ANNAMNEEDI

Emmanuel Pecnard

Gilles BRUNEAU

Lucile Drobecq

Anne-Charlotte Trouillet

https://orcid.org/0000-0003-3268-7858

Romain YVINEC

Gaëlle Lefort

INRAE https://orcid.org/0000-0001-8166-2281

Pascale Crepieux

Physiologie de la Reproduction et des Comportements (PRC)

Angela Sirigu

Centre de Neuroscience Cognitive, Centre National de la Recherche Scientifique, 69675 Bron, France

Dietmar Kuhl

Ora Ohana

University Medical Centre Hamburg-Eppendorf

Pablo Chamero
CNRS, INRAE, University of Tours https://orcid.org/0000-0002-4305-1073

Article

Keywords: Arc/Arg3.1, Neomycin cassette, knockout mouse, maternal behavior, social behavior, oxytocin, synaptic plasticity

Posted Date: July 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10129888/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors have declared there is **NO** conflict of interest to disclose

Abstract

The Neomycin resistance cassette (Neo$^+$) is commonly inserted in the genome of mice to generate knock-out (KO) models. The effect of gene deletion on social behaviors in mice is inconsistent across studies using Neo$^+$ and Neo$^-$ lines, particularly *Arc/Arg3.1* KO models. In this study, we identified severe impairments in maternal behavior in Neo$^+$, but not Neo$^-$ *Arc/Arg3.1* KO dams. These deficits were associated with reduced sociability and abnormal social information processing in Neo$^+$ *Arc/Arg3.1* KO dams, and were further exacerbated by impaired social communication in their pups. Mechanistically, although the Neo cassette product was not cytotoxic, its presence altered ERK signaling, gene expression profile, and the oxytocin system. However, oxytocin administration failed to rescue social deficits in Neo$^+$ *Arc/Arg3.1* KO animals. Notably, early-life social enrichment improved social interaction with familiar conspecifics, but did not restore responses to unfamiliar animals or maternal behavior. Together, our findings demonstrate that the Neo cassette influence behavioral phenotypes, particularly social and maternal behavior in *Arc/Arg3.1* KO mice, highlighting the need to carefully re-evaluate conclusions drawn from animal models retaining the Neo cassette in neuroscience research.

INTRODUCTION

The ability of the mammalian brain to learn and adapt to new environments relies on synaptic plasticity[1]. The activity-regulated cytoskeleton-associated protein (*Arc*), also known as activity-regulated gene of 3.1 kilobases (*Arg3.1*), is a key regulator of long-lasting synaptic plasticity, such as long-term potentiation and depression[2–4]. *Arc/Arg3.1* is an immediate early gene (IEG) predominantly expressed in excitatory glutamatergic neurons within the hippocampus, cortex, and striatum[5,6]. In response to stimuli, *Arc/Arg3.1* mRNA is rapidly transcribed within minutes, and undergoes activity-dependent translation at post-synaptic sites in dendrites[7]. Disruption of *Arc/Arg3.1* impairs learning, long-term memory consolidation, and sensory-motor gating, while its dysregulation affects cognitive flexibility, highlighting its essential role in synaptic plasticity[8,9,4].

In humans, *ARC/ARG3.1* gene has been linked to alcohol use disorder[10] and neurodevelopmental and psychiatric disorders characterized by social deficits[11]. Notably, 43 *ARC/ARG3.1* single nucleotide polymorphisms have been associated with schizophrenia[12]. Additionally, increased plasma levels of ARC/ARG3.1 have been described in children with ASD[13]. Elevated *Arc/Arg3.1* levels have been reported in mouse models of Fragile X and Angelman syndromes, both associated with autism spectrum disorders (ASD)[14,15]. However, studies using *Arc/Arg3.1* knockout (KO) mice have yielded inconsistent results: one study reported reduced sociability and social novelty in the first KO1 mouse line[16], while the second found no effect on sociability using a different KO2 line[17]. As highlighted by Gao *et al.*[17], the key difference between these lines is the insertion of the Neomycin resistance cassette (Neo$^+$) in the genome of the KO1 line[16]. Whether the presence of Neo$^+$ in the *Arc/Arg3.1* locus has an impact on behavioral phenotypes is currently unknown.

In this study, we aimed to clarify whether inconsistencies in reported social behaviors of *Arc/Arg3.1* KO mice arise from the Neo cassette insertion. We examined social, olfactory, and non-social behaviors in the Neo^+ and Neo^- *Arc/Arg3.1* KO1 mouse line. Our findings revealed significant behavior impairments in Neo^+, but not in Neo^- KO mice. The Neo cassette product induced changes in cellular signaling and gene expression, particularly affecting the oxytocin system. Overall, our findings reveal a strong influence of the Neo cassette on behavioral phenotypes in *Arc/Arg3.1* KO mice.

MATERIALS AND METHODS

For a detailed description, please see supplementary materials and methods.

Animals

All mouse breeding, care, and experimental procedures followed European, French and German Directives and were approved by the local ethics committees – CEEA Val de Loire N°19 and the French Ministry of Teaching, Research and Innovation (APAFIS #18035-2018121213436249), and the animal welfare office of the city of Hamburg and the animal care committee at the University Medical Center Hamburg-Eppendorf. The first line used in this study included WT and *Arc/Arg3.1* KO1 mice carrying the Neo cassette (Neo^+ KO1)[16,18] (JAX stock #007662). Neo^- KO1 mice were generated by excising the Neo cassette from the genome through the Flox sites of Neo^+ KO1 mice (**Figure S1**, supplementary materials and methods), all bred on a mixed 50% C57BL/6J – 50% 129S2 background[19]. The second line comprised WT and *Arc/Arg3.1* KO mice lacking the Neo cassette (Neo^- KO2), bred on a C57BL/6J background[4]. KO1 and KO2 lines were housed in separate animal facilities under the same conditions: a 12-hour light/dark cycle, with food and water ad libitum, controlled temperature (21°C) and humidity (50%), housed in social groups of 2–4 animals. All experimental mice were two months old sexually naïve males and females at the start of the behavioral experiments.

Behaviors

Behavioral tests were conducted at the start of the light phase in WT, Neo^+ and Neo^- KO1 lines, with one test per day. Sociability was tested in the three-chambered test and social interaction in the automatic Live Mouse Tracker (LMT) device[20] with up to four familiar (cage mates) and unfamiliar, age-, sex-matched conspecifics as described previously[21]. Social memory was evaluated in the social recognition test. Olfaction was assessed over several assays with neutral, food, and social odors. Perseverative, stereotyped, and compulsive behaviors were tested in the spatial Y-maze task, motor stereotypy, and marble burying tests, respectively. Locomotion was assessed in the open-field test and motor coordination in the string test. Anxious-like behavior was evaluated in the elevated-plus maze, novelty-suppressed feeding, and open field tests. Spatial memory was assessed in object location recognition and Y-maze tasks, while the recognition memory was evaluated in the novel object recognition task. Maternal behavior using the pup retrieval and nest-building tests was assessed in 3-4-month-old

primiparous dams following 2 weeks breeding with males, followed by one week isolation and parturition. The pup retrieval assay was performed only in WT and Neo$^-$ KO2 line. Ultrasound vocalizations following maternal separation were recorded in pups, along with the assessment of their social skills in the homing test, and their gripping, cliff avoidance, and righting reflexes in the respective tests. All animal research has been performed and reported according to the ARRIVE guidelines[22].

Quantitative PCR (qPCR)

The expression of oxytocin family genes (*Oxt*, *Oxtr*, *Avp*, *Avpr1a*), immediate early genes (*Homer1a*, *Fos*, *Egr1*, *Foxp1*), ASD-associated genes (*Shank3*, *Fmr1*, *Cpeb4*, *Cntnap2*), neuropeptides genes (*Cck*, *Gal*, *Cartpt*, *Esr1*), and housekeeping gene *Gapdh* (**Table S6**) was investigated in the prefrontal cortex (PFC), nucleus accumbens (NAC), caudate putamen (CPU), paraventricular (PVN), and supraoptic (SON) nuclei of three-months-old WT, Neo$^+$ KO1, and Neo$^-$ KO1 dams, and two-month-old naïve WT and Neo$^+$ KO1 mice.

RNA sequencing

Bulk RNA sequencing (RNA-seq) was performed in the PVN of two-month-old naïve WT, Neo$^+$ KO1, and Neo$^-$ KO1 females dissected after social interaction with genotype-matched littermates. Following total RNA extraction, oligo(dT) enrichment, library preparation and strand-specific NovaSeq sequencing (2x150 bp, > 20M reads per sample), reads were aligned to the mouse genome (GRCm39) with STAR[23] and transcripts were quantified and normalized using SALMON[24] and DESeq2[25]. Differentially expressed genes were visualized and analyzed using volcano plot (ggplot2), heatmap (pheatmap R)[26], gene ontology (gprofiler2[27], STRINGdb[28] and rbioapi[29] R package) and curated from autism (AutDB, SFARI) and schizophrenia (SZDB) databases.

Arc/Arg3.1 genomic environment interaction

Cis- and *trans*-, short- and long-range 3D genomic interactions of the murine *Arc/Arg3.1* locus and its topologically associated domain (TAD) on chromosome 15 were explored using single-cell Hi-C data from neuronal subtypes in the 3D Genome Browser dataset[30].

Western blot

Protein levels of ERK1/2, phospho-ERK1/2, Arc, the Neo product (aminoglycoside phosphotransferase) and GAPDH were assessed in the PVN and OB of two-month-old naïve WT, Neo$^+$ KO1, and Neo$^-$ KO1 females (males in Figure S11A).

Immunohistochemistry

Brains were collected from WT and Neo$^+$ KO1 dams on pups' postnatal day 14 to immunolabel oxytocin pre-pro-peptide and Neo product, or from WT and Neo$^+$ KO1 naïve mice to co-label oxytocin pre-pro-peptide and Arc.

Chronic oxytocin treatment and social enrichment

Two-month-old naïve WT and Neo$^+$ KO1 mice were intranasally injected daily for 28 days with a solution of 20 µg kg^{-1} (0.03 IU) oxytocin (OT) or saline (NaCl 0.9%) as control. On days 0, 7, 14, 21, and 28 of treatment, mice were subjected to social interaction in the LMT with up to four unfamiliar, age-, sex-, and treatment-matched conspecifics to evaluate the effect of OT treatment on social interaction and on day 25 for motor stereotypies. For early-life social enrichment experiment, following heterozygous breeding, Neo$^+$ KO1 mice were weaned and raised at 1:4–1:2 ratio with WT and heterozygous littermates and tested in the LMT at two months of age.

Statistical analysis

All data and statistical analyses were performed using R software (version 4.3.1). For a detailed description, please see Figure legends and supplementary materials and methods. Raw data, including western blots and microscopy images are available at https://doi.org/10.5281/zenodo.20589372, while mean ± standard deviation (sd), n and statistics are reported in **Tables S1-S5**. RNAseq data are available at ENA (PRJEB112177 (ERP192761) project accession number).

RESULTS

Neo$^+$ KO1 dams display maternal behavior impairments

To investigate the impact of Neo insertion in the *Arc/Arg3.1* locus on social behaviors, we examined maternal behavior in dams from Neo$^+$ *Arc/Arg3.1* KO (Neo$^+$ KO1), which previously showed sociability impairments[16], and Neo$^-$ *Arc/Arg3.1* KO1 mice with the Neo cassette excised from the genome (Neo$^-$ KO1) derived from the same line (**Figure S1**).

Remarkably, 93% of Neo$^+$ KO1 dams failed to retrieve their Neo$^+$ KO1 pups within 30 min of the pup retrieval test, resulting in a longer pup retrieval latency compared to WT and Neo$^-$ KO1 dams (Fig. 1A-D). Neo$^+$ KO1 dams displayed maternal care impairment (reduced time crouching over the pups), while their ability to detect their pups was intact (**Figure S2A-B, Table S1**). We confirmed these results using the second Neo$^-$ *Arc/Arg3.1* KO line (Neo$^-$ KO2), which previously exhibited no sociability impairments[17]. Like Neo$^-$ KO1 dams, Neo$^-$ KO2 dams displayed no differences in maternal behavior, compared with their respective WT (Fig. 1E-F, **Figure S2C**). Differences between WT mice from the two lines reflected their

distinct genetic backgrounds, consistent with previous report showing enhanced sociability in mixed C57BL/6J;129S2 *vs.* inbred C57BL/6J mice[19].

Importantly, to exclude that these observations might result from social impairments in KO pups[31], we tested Neo^{+} KO1 dams with heterozygous Neo$^{+/-}$ *Arc*$^{+/-}$ (Neo^{+} *Arc*$^{+/-}$) pups. Neo^{+} KO1 dams still displayed impaired maternal behavior (longer pup retrieval latency) compared to WT dams with heterozygous Neo^{+} *Arc*$^{+/-}$ pups (Figs. 1G-H, S2D), though to a lesser extent than when tested with homozygous Neo^{+} (Neo$^{+/+}$) KO1 pups (Fig. 1A-B). Additionally, Neo^{+} *Arc*$^{+/-}$ dams showed maternal deficits comparable to Neo^{+} KO1 with heterozygous Neo^{+} *Arc*$^{+/-}$ pups (Figs. 1G-H).

We found no differences in parturition delay, litter size, lactation, or percentage of pup survival compared to WT controls in all lines (**Table S1**).

In conclusion, insertion of a single Neo cassette allele in the *Arc/Arg3.1* locus is sufficient to induce maternal behavior impairments in dams, an effect that is further influenced by the genotype of the pups.

Neo cassette affects sociability in both dams and pups

Next, we verified whether maternal impairments were accompanied with social deficits. Neo^{+} KO1 mice displayed reduced social interaction (nose contact) and motivation (social approach) with both familiar (cage mates) and unfamiliar conspecifics, compared to WT and Neo^{-} KO1 mice, particularly in females (Figs. 2A-B, S3A-B, **Table S2**). Importantly, Neo^{-} KO1 males displayed increased social interaction with unfamiliar conspecifics, compared with WT males, suggesting that the insertion of the Neo masked this phenotype (**Figure S3B**). We excluded that poor maternal care provided by Neo^{+} KO1 dams did cause these impairments, since Neo^{+} *Arc*$^{+/-}$ mice raised by either WT or Neo^{+} KO1 dams did not exhibit social interaction impairments (Figs. 2C, S3C-D). Sociability impairments in Neo^{+} KO1 mice were further confirmed in the sociability phase of the three-chambered test and were not attributable to social memory deficits (**Figure S4**).

Since maternal and social behaviors in rodents heavily rely on olfaction[32], we evaluated potential olfactory deficits in Neo^{+} KO1 mice. These mice showed no obvious impairments in olfactory detection of neutral, food, or predator odors, but spent less time sniffing social odors in the habituation-dishabituation test (**Figure S5A-D, Table S1**). This suggests intact general odor detection, but potential deficits in social odor discrimination or motivation. To further evaluate potential alterations in social odor processing, we performed a social dominance test, which is largely mediated by olfactory sensing[33,34]. Both dominant and subordinate Neo^{+} KO1 mice displayed increased social dominance facing dominant WT mice (**Figure S5E**). Furthermore, Neomycin protein product was not detected in the vomeronasal organ (VNO), main olfactory epithelium (MOE) and olfactory bulb (OB) (Figs. 3D, S5F-G). Collectively, these results indicate that the Neo cassette may impair social odor-induced interest, but not odor detection or discrimination.

As Neo^{+} KO1 dams displayed worse maternal impairment with Neo^{+} KO1 pups, we also assessed social communication in pups. Neo^{+} KO1 pups exhibited shorter total time vocalizing, as well as delayed latency to vocalize, fewer and shorter ultrasound vocalizations following maternal separation compared to WT and Neo^{+} *Arc*$^{+/-}$ pups (Fig. 2D-E, **Table S3**). Additionally, both Neo^{+} KO1 and Neo^{+} *Arc*$^{+/-}$ pups exhibited delayed latency to reach the nest area and spent less time in the nest bedding than WT pups in the olfactory-based homing test[35] (**Figure S6A-B**), suggesting early social impairments in both genotypes. Importantly, no differences were observed in three reflex assays, indicating that these impairments did not result from motor deficits (**Figure S6C**).

We also evaluated non-social behaviors that could influence sociability, such as distinct memory, anxious-like, perseverative or compulsive behaviors, and motor coordination (Figs. 2F-G, S7-8, **Table S1**). Neo^{+} KO1 mice displayed mild spatial memory impairment as previously reported[16], while Neo^{-} KO1 mice showed no difference in short- and mid-term social or object recognition memory (**Figure S4B and S7**). Interestingly, both Neo^{+} KO1 and Neo^{-} KO1 mice did not show differences in anxious-like behavior in the elevated-plus maze and open field tests. By contrast, increased immobility during exploration of a novel environment was observed exclusively in Neo^{-} KO1 mice (Figs. 2F, S8A), suggesting that the Neo cassette may have masked this phenotype associated with *Arc/Arg3.1* deletion. Furthermore, both Neo^{+} and Neo^{-} KO1 mice displayed increased self-grooming, with no difference in restrained or compulsive behaviors or motor coordination (Figs. 2G, S8B-C).

In conclusion, the Neo cassette induced social deficits in both females and pups, along with communication impairments in pups, worsening maternal behavior deficits in dams.

Neo cassette perturbs gene expression, signaling, and the oxytocin system

Next, we investigated the molecular mechanisms underlying the behavioral effects of the Neo cassette. Although insertion of the Neo cassette into the large Shank3 gene induces alternative splicing and the formation of fusion proteins[36,37], we did not detect either in Arc/Arg3.1 KO mice (Figs. 3A-D). RNA-seq analysis revealed decreased Arc mRNA levels in the PVN of Neo^{+} KO1 mice, which were attributed to reduced read mapping within the Arc/Arg3.1 gene (Fig. 3B-C, Figure S1). To detect potential alterations on the expression of nearby genes[38,39] following insertion of the Neo cassette at the Arc/Arg3.1 locus on chromosome 15, we examined the local genomic environment using RNA-seq and 3D genomic interaction analyses[30]. These analyses revealed that the expression of all short- and long-range expressing genes was not affected by the insertion of the Neo cassette (Fig. 3E-G).

While mainly reported in glutamatergic neurons of the hippocampus, cortex, and striatum[5,6], we found that Arc was expressed in oxytocinergic (OT) neurons of the paraventricular nucleus of the hypothalamus (PVN), a critical region for regulating maternal and social behaviors[32], but not in Neo^{+} KO1 mice (Fig. 4A). Strikingly, we detected expression of the Neo cassette protein product in the PVN, but not

in other brain structures of Neo+ KO1 dams (Figs. 4B, S9). Although we found a comparable number of OT neurons in the PVN and supraoptic nucleus (SON), OT fiber density was reduced in the nucleus accumbens (NAC) and prefrontal cortex (PFC) compared to WT (Figs. 4C, S10). These findings suggest that the Neo cassette affected OT neuronal connectivity, rather than inducing cytotoxicity.

As Neo product is a bacterial aminoglycoside phosphotransferase structurally resembling eukaryotic kinases[40], we hypothesized that it might affect ERK1/2 phosphorylation, a cell signaling integrator involved in neurodevelopment[41,42]. In the PVN, phosphorylated ERK1/2 levels were increased in Neo^+ KO1 females compared to WT and Neo^- KO1 females, but not in males or in the OB (Figs. 4D, S11A-B, **Table S4**). Moreover, Neo expression in HEK293 cells was sufficient to selectively increase phosphorylated ERK1/2, but not rpS6 (**Figure S11C, Table S4**), suggesting that the Neo cassette enhances ERK1/2 signaling.

Since the ERK pathway regulates gene expression[41,42], we performed RNA-seq in the PVN of WT, Neo^+ and Neo^- KO1 females (Figs. 4E-F). We identified 19 downregulated genes and 6 upregulated genes in Neo^+ KO1 females (none located on chromosome 15). Those genes were associated with reduced protein synthesis (e.g., *Acp1*, *Rpl34*, *Hddc3*, *Hspa8*), glial potassium buffering linked to seizure susceptibility (*Kcnj10*), as well as increased ubiquitin activity (*Sik1*, *Mid1*), neuronal oxidative stress (*Crym*) and DNA damage (schizophrenia-associated *Ints7*), and dysregulation in extracellular matrix components (*Col6a1* and I*tga10*) and transcription regulation (*Zkscan8*, *Hspa8*, *Hmga1b*). Moreover, we integrated oxytocin, vasopressin, and galanin genes regulating maternal and social behaviors[43], ASD-associated genes, and synaptic plasticity IEGs in the PVN, SON, PFC, NAC, and caudate putamen (CPU) expression, with maternal and social behaviors and motor stereotypies (**Figure S12, Tables S1-2, S5**). Neo^+ KO1 dams segregated apart from Neo^- KO1 or WT dams in both behavior and gene expression (**Figure S12A**). Dysregulated *Oxtr* in the PFC and *Shank3* in the SON were correlated with longer pup retrieval latency, along with *Cck* and *Oxtr* in the PVN, suggesting a potential link between maternal impairments and reduced OT signaling. Furthermore, we also identified *Oxtr* dysregulation across brain regions in naïve Neo^+ KO1 males and females (**Figure S12B**). Social interaction deficits correlated with IEG dysregulation, particularly *Homer1a* (**Figure S12B**). Conversely, *Arc/Arg3.1* deletion had a mild effect, with only two dysregulated genes in the PVN and a potential correlation between ASD-associated gene *Cntnap2* expression and increased self-grooming (**Figure S13**).

Given the OT system dysregulation, we investigated whether acute (day 0) or subchronic (day 1–28) intranasal OT administration at 20 μg kg^{-1} could alleviate sociability impairments in Neo^+ KO1 mice. OT treatment did not improve social interactions with unfamiliar conspecifics or reduce stereotyped behaviors in Neo^+ KO1 mice (Figs. 5A-B, S14, **Table S2**), suggesting that the Neo cassette-induced OT dysregulation cannot be counteracted by OT administration. By contrast, OT treatment increased social interaction in WT mice by day 28 (Figs. 5B, S15C), validating the efficacy of OT administration.

In summary, the Neo cassette induced changes in gene expression, oxytocin system dysregulation and, ultimately, maternal and social deficits that cannot be reversed by OT administration.

Social enrichment partially improves sociability deficits in Neo$^+$ KO1 mice

Although Neo$^+$ KO1 mice initially showed sociability impairments, repeated exposure to unfamiliar conspecifics reduced genotype differences by day 14 in saline (SAL)-treated mice (Fig. 5C). A similar effect was observed in OT-treated Neo$^+$ KO1 mice, although to a lesser extent and delayed to day 28 (Fig. 5C-D). This effect did not result from changes in anxious-like behavior, as no difference in stretch-attended posture (SAP) was observed over the trials (**Figure S14E-F**), suggesting a potential effect of the social environment on sociability in Neo$^+$ KO1 mice.

To further investigate this, we tested whether an enriched early-life social environment within the critical post-natal period[21] would reverse the sociability and maternal impairments in Neo$^+$ KO1 mice by raising them with socially-competent WT and heterozygous Neo$^+$ and Neo$^-$ *Arc*$^{+/-}$ cage mates. Remarkably, social enrichment improved sociability in Neo$^+$ KO1 mice when interacting with familiar (cage mates), but not unfamiliar conspecifics (Fig. 5E-F, **Table S2**). Although WT mice displayed increased total nose contacts in the enriched social environment, promoting more dyadic contacts with littermates, Neo$^+$ KO1 mice showed enhanced social approach and nose-anogenital contacts, indicating greater social interest (Figs. 5E-F, S15). However, an enriched early-life social environment did not reverse maternal impairments in Neo$^+$ KO1 dams with Neo$^+$ *Arc*$^{+/-}$ pups (Fig. 5G), indicating that this behavior cannot be improved by social enrichment, which is consistent with the Neo cassette's negative effect in heterozygous dams.

In conclusion, repeated social interactions and enriched early-life social environment partially rescued sociability impairments, but not maternal behavior in Neo$^+$ KO1 mice.

DISCUSSION

In this study, we demonstrated that the insertion of the Neo cassette in the *Arc/Arg3.1* locus led to significant impairments in maternal behaviors, as well as social and non-social behaviors. These results were confirmed by the absence of these impairments in two different lines of Neo$^-$ KO mice. Notably, no impairments were observed in Neo$^-$ KO1 derived from the Neo$^+$ KO1 line (retaining all other genomic elements, such as EGFP under the control of the *Arc/Arg3.1* promoter). We identified that the severe maternal impairments caused by the Neo cassette were associated with social deficits in females. Although maternal care is vital in shaping social behaviors[44,45], impaired maternal care of Neo$^+$ KO1 dams had no impact on the sociability of their adult offspring, ruling it out as a confounding factor in social deficits. The presence of the Neo cassette in *Arc/Arg3.1* KO mice also induced core social ASD-like traits – impairments in social communication and interaction.

Additionally, the short-term spatial memory impairment reported here and previously in Neo^{+} KO1 mice[16] was not found after Neo cassette excision. These findings indicate that the Neo cassette, rather than *Arc/Arg3.1* deletion, may contribute to deficits in hippocampus-dependent short-term memory[4]. This aligns with the intact short-term memory observed in the Neo^{-} KO2 mouse line[4,46]. However, it is well-established that *Arc/Arg3.1* deletion or downregulation strongly suppress long-term memory[46–48]. Nevertheless, *Arc/Arg3.1* deletion alone induced exacerbated stereotyped behaviors (self-grooming), which are potentially correlated with increased ASD-associated gene *Cntnap2* expression in Neo^{-} KO1 mice. However, previous findings reported increased self-grooming in *Cntnap2* KO mice[49], suggesting that the relationship between *Cntnap2* expression and stereotyped behaviors may be complex and warrants further investigation. Notably, *Arc/Arg3.1* deletion increased social interactions in Neo^{-} KO1 males and novelty-induced immobility in males and females. These effects were not observed in Neo^{+} KO1 mice, indicating that Neo cassette insertion both induced and masked specific behavioral phenotypes.

Maternal impairments induced by the Neo cassette likely stem from deficits in social motivation. Impaired responses to social odors in Neo^{+} KO1 mice did not originate from alterations in the olfactory periphery as Neo is absent in the olfactory epithelia. Even if gene expression changes in these epithelia have been previously reported after Neo cassette insertion[50], social olfactory discrimination deficits likely arise from central olfactory processing and/or reduced motivation to sniff social odors.

Neo expression was detected only in the PVN of Neo^{+} KO1 mice. Because Neo is a bicistronic element flanked by loxP sites that may form secondary structures in the GFP/Neo/Arc transcript of Neo^{+} KO1 mice, we cannot exclude low levels of expression or translation in other neurons or brain structures. The PVN undergoes pronounced synaptic plasticity during gestation and postpartum[51], projects to brain regions of the socio-emotional circuit, including the NAC, PFC and hippocampus, and exhibits sexual dimorphism[52]. Thus, Neo expression restricted to the PVN may underlie the social and maternal impairments observed in Neo^{+} KO1 females.

The molecular mechanisms through which the Neo cassette affects social behaviors were primarily linked to reduced protein synthesis and altered cell adhesion in the PVN, suggesting a global cellular stress response, and impaired signaling and synaptic plasticity (*Oxtr* and *Homer1a*, respectively). While Neo cassette insertion can alter nearby gene expression[38,39], no such dysregulation was observed in genes in chromosomal proximity to the *Arc/Arg3.1* locus. Importantly, Neo expression enhanced ERK1/2 signaling in both the PVN and heterologous cells. ERK dysregulations are reported in various neuropsychiatric conditions, including autism and schizophrenia[53,54]. Moreover, enhanced ERK signaling has been linked with impaired synaptic plasticity, learning, memory, and social interactions[42] and autistic-like behavioral phenotypes in mice[55], and leads to abnormal gene expression and neuronal development[41,42]. Consistent with this, we also observed in Neo^{+} KO1 mice both abnormal gene expression and connectivity of OT neurons to the PFC and NAC, structures controlling social reward and

interactions[56,57]. Although the OT system is closely linked to maternal behavior[43], OT treatment did not reverse the behavioral impairments in Neo$^+$ KO1 mice, suggesting an underlying sustained OT signaling dysregulation. In a neonatal maternal separation model of autism in rats, OT treatment rescued both social phenotype and ERK activation[58]. Those findings suggest that OT treatment may be ineffective when the ERK pathway is overactivated, as observed in Neo$^+$ KO1 females.

OT treatment followed by repeated exposure to conspecifics enhanced social interaction in WT, whereas this effect was absent in Neo$^+$ KO1 mice. Social interaction impairments in Neo$^+$ KO1 mice were partially improved by repeated social interactions with genotype-matched unfamiliar conspecifics and early-life social enrichment. In particular, early social environment enrichment improved social interest in Neo$^+$ KO1 mice, likely due to constant stimulation from socially competent littermates. Impairments in naïve Neo$^+$ KO1 mice were linked to dysregulated IEG expression, particularly *Homer1a* as reported previously[59], which plays a critical role in brain plasticity and adaptation to novel environments[60], and is altered in multiple mouse models of ASD displaying social impairments[59,61]. Therefore, repeated and long-lasting social stimulation may partially restore synaptic plasticity *via* enhanced IEG expression, relieving the negative impact of Neo cassette on social interactions.

Overall, our results show that the Neo cassette exerts off-target effects, that induced or masked behavioral phenotypes in *Arc/Arg3.1* KO mice. Our findings have major implications for phenotypes in KO mouse models that retain the Neo cassette, potentially misleading the interpretation of gene function in other Neo$^+$ mouse models, and depending on Neo expression patterns. For instance, difference in exon deletion in *Shank3* KO lines[62], variations in experimental conditions across laboratories, and Neo cassette expression may contribute to the phenotypic variability observed in Neo$^+$ and Neo$^-$ *Fmr1* and *Shank3* KO mice[59,62–67]. Additionally, the Neo cassette may contribute to the maternal behavior impairments in Neo$^+$ *Fosb*[68] and *Cd38* KO mice[69] or social interaction and/or communication phenotypes of numerous KO mouse models of ASD, including Neo$^+$ *Shank3*, *Cntnap2*, *Ube3a*, *Nlgn1-3*, *Nrxn1*, *Pten*, and *Cd38* KO mice[63,69–74]. Furthermore, through expression in the olfactory epithelia and their projecting areas[50], Neo cassette may also induce olfactory impairments reported in several mouse models of ASD[32], impacting olfactory-dependent social behaviors. The local genomic environment may influence Neo product expression or its effect on nearby gene regulation, leading to variable behavioral effects across Neo$^+$ KO lines.

In conclusion, the Neo cassette exerts detrimental effects on behaviors in *Arc/Arg3.1* KO mice. Our results advocate for re-evaluating behavior phenotypes in KO mouse models carrying the Neo cassette, especially for conditions characterized by social and maternal impairments. This re-evaluation could facilitate the predictive validity of KO models, enhancing their relevance in preclinical studies, and improving translatability to humans.

ABBREVIATIONS

ASD autism spectrum disorder

BNST bed nucleus of stria terminalis

CPU caudate putamen

IEG immediate early gene

KO knock-out

LMT Live Mouse Tracker

MPOA medial preoptic area

NAC nucleus accumbens

Neo neomycin

OB olfactory bulb

OT oxytocin

PFC prefrontal cortex

PVN paraventricular nucleus of the hypothalamus

RNA-seq RNA sequencing

SAL saline

SON supraoptic nucleus

WT wild-type

Declarations

CONFLICT OF INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

AD, ACT, HE, DK, PC, OO, and LPP designed experiments; AD, AHAK, AA, HE, EP, LD, HV, and LPP performed experiments; AD, AHAK, AA, EP, LD, HV, GB, and LPP contributed to the data collection; AD, EC, OM, NA, GL, BP, PC, RY, OO, and LPP contributed to data analysis; AD performed all statistical analysis

and data integration; AD and LPP wrote the original drafts; LPP, AS, OO and DK contributed to the funding acquisition; LPP contributed to project conceptualization and supervision.

ACKNOWLEDGEMENTS

Mouse breeding and care were performed at PAO, the rodent INRAE Animal Physiology Facility (https://doi.org/10.15454/1.5573896321728955E12). We thank Eva Kronberg from the UKE mouse facility at the ZMNH for breeding Neo⁻ *Arc/Arg3.1* KO2 mice and assistance with the experiments. This work has benefited from the equipment and expertise of the Imaging facility "Plateau d'Imagerie Cellulaire" (PIC) of UMR-PRC (http://doi.org/10.17180/arap-gj59). We sincerely thank Dr. Emmanuel Valjent for his valuable suggestions on the manuscript. During the preparation of this work, we used ChatGPT, developed by OpenAI, in order to improve English editing. After using this tool, we reviewed and edited the content as needed and take full responsibility for the content of the publication.

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 851231). OO, DK and AK were supported by a grant from the Schaller-Nikolic foundation to OO and DK. LPP and AD acknowledge the LabEx MabImprove (grant ANR-10-LABX-53-01) for the financial support of AD PhD's co-fund. The funders played no role in the design, analysis, or interpretation of the study's data.

References

1. Citri A, Malenka RC. Synaptic plasticity: Multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 2008; 33: 18–41.
2. Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U *et al.* Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proceedings of the National Academy of Sciences* 1995; 92: 5734–5738.
3. Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG *et al.* Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 1995; 14: 433–445.
4. Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C *et al.* Arc/Arg3.1 Is Essential for the Consolidation of Synaptic Plasticity and Memories. *Neuron* 2006; 52: 437–444.
5. Klebaur JE, Ostrander MM, Norton CS, Watson SJ, Akil H, Robinson TE. The ability of amphetamine to evoke arc (Arg 3.1) mRNA expression in the caudate, nucleus accumbens and neocortex is modulated by environmental context. *Brain Research* 2002; 930: 30–36.
6. Vazdarjanova A, Ramirez-Amaya V, Insel N, Plummer TK, Rosi S, Chowdhury S *et al.* Spatial exploration induces ARC, a plasticity-related immediate-early gene, only in calcium/calmodulin-dependent protein kinase II-positive principal excitatory and inhibitory neurons of the rat forebrain. *Journal of Comparative Neurology* 2006; 498: 317–329.

7. Eriksen MS, Bramham CR. Molecular physiology of Arc/Arg3.1: The oligomeric state hypothesis of synaptic plasticity. *Acta Physiologica* 2022; 236: 1–20.
8. Nikolaienko O, Patil S, Eriksen MS, Bramham CR. Arc protein: a flexible hub for synaptic plasticity and cognition. *Seminars in Cell and Developmental Biology* 2018; 77: 33–42.
9. Wall MJ, Collins DR, Chery SL, Allen ZD, Pastuzyn ED, George AJ *et al.* The Temporal Dynamics of Arc Expression Regulate Cognitive Flexibility. *Neuron* 2018; 98: 1124–1132.e7.
10. Pagano R, Salamian A, Zielinski J, Beroun A, Nalberczak-Skóra M, Skonieczna E *et al.* Arc controls alcohol cue relapse by a central amygdala mechanism. *Molecular Psychiatry* 2023; 28: 733–745.
11. American Psychiatric Association. *Diagnostic and statistical manual of mental disorders (5th ed.)*. 5th editio. 2013 doi:https://doi.org/10.1176/appi.books.9780890425596.
12. Chuang Y-A, Hu T-M, Chen C-H, Hsu S-H, Tsai H-Y, Cheng M-C. Rare mutations and hypermethylation of the ARC gene associated with schizophrenia. *Schizophrenia Research* 2016; 176: 106–113.
13. Alhowikan AM. Activity-Regulated Cytoskeleton-Associated Protein Dysfunction May Contribute to Memory Disorder and Earlier Detection of Autism Spectrum Disorders. *Medical Principles and Practice* 2016; 25: 350–354.
14. Niere F, Wilkerson JR, Huber KM. Evidence for a Fragile X Mental Retardation Protein-Mediated Translational Switch in Metabotropic Glutamate Receptor-Triggered Arc Translation and Long-Term Depression. *The Journal of Neuroscience* 2012; 32: 5924–5936.
15. Pastuzyn ED, Shepherd JD. Activity-Dependent Arc Expression and Homeostatic Synaptic Plasticity Are Altered in Neurons from a Mouse Model of Angelman Syndrome. *Frontiers in Molecular Neuroscience* 2017; 10. doi:10.3389/fnmol.2017.00234.
16. Managò F, Mereu M, Mastwal S, Mastrogiacomo R, Scheggia D, Emanuele M *et al.* Genetic Disruption of Arc/Arg3.1 in Mice Causes Alterations in Dopamine and Neurobehavioral Phenotypes Related to Schizophrenia. *Cell Rep* 2016; 16: 2116–2128.
17. Gao X, Grendel J, Muhia M, Castro-Gomez S, Susens U, Isbrandt D *et al.* Disturbed prefrontal cortex activity in the absence of schizophrenia-like behavioral dysfunction in arc/arg3.1 deficient mice. *Journal of Neuroscience* 2019; 39: 8149–8163.
18. Wang KH, Majewska A, Schummers J, Farley B, Hu C, Sur M *et al.* In Vivo Two-Photon Imaging Reveals a Role of Arc in Enhancing Orientation Specificity in Visual Cortex. *Cell* 2006; 126: 389–402.
19. Dudas A, Novak A, Gora C, Pecnard E, Azzopardi N, Morisset-Lopez S *et al.* Mixed genetic background better recapitulates developmental and psychiatric phenotypes and heterogeneity than inbred C57BL/6J mice. *Sci Rep* 2025; 15: 39130.
20. de Chaumont F, Ey E, Torquet N, Lagache T, Dallongeville S, Imbert A *et al.* Real-time analysis of the behaviour of groups of mice via a depth-sensing camera and machine learning. *Nature Biomedical Engineering* 2019; 3: 930–942.
21. Gora C, Dudas A, Court L, Annamneedi A, Lefort G, Nakahara TS *et al.* Effect of the social environment on olfaction and social skills in wild-type and a mouse model of autism. *Translational Psychiatry* 2024; 14: 464.

22. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M *et al.* The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLOS Biology* 2020; 18: e3000410.
23. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; 29: 15–21.
24. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 2017; 14: 417–419.
25. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15: 550.
26. Kolde R. pheatmap: Pretty Heatmaps. 2010;: 1.0.13.
27. Kolberg L, Raudvere U, Kuzmin I, Adler P, Vilo J, Peterson H. g:Profiler—interoperable web service for functional enrichment analysis and gene identifier mapping (2023 update). *Nucleic Acids Research* 2023; 51: W207–W212.
28. Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F, Hachilif R *et al.* The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic Acids Research* 2023; 51: D638–D646.
29. Rezwani M, Pourfathollah AA, Noorbakhsh F. rbioapi: user-friendly R interface to biologic web services' API. *Bioinformatics* 2022; 38: 2952–2953.
30. Yu S, Fu Y, Wong JH-Y, Wang J, Zhao H, Zhao J *et al.* The 3D Genome Browser 2.0: an enhanced online platform for visualizing and analyzing 3D genome architecture. *Nucleic Acids Res* 2026; 54: D48–D54.
31. Kuroda KO, Tachikawa K, Yoshida S, Tsuneoka Y, Numan M. Neuromolecular basis of parental behavior in laboratory mice and rats: With special emphasis on technical issues of using mouse genetics. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 2011; 35: 1205–1231.
32. Dudas A, Nakahara TS, Pellissier LP, Chamero P. Parenting behaviors in mice: Olfactory mechanisms and features in models of autism spectrum disorders. *Neuroscience & Biobehavioral Reviews* 2024; 161: 105686.
33. Borak N, Wai P, Rodriguez Villamayor P, Claxton PM, Liang S, Kohl J. Dominance rank inference in mice via chemosensation. *Current Biology* 2025; 35: 2746–2751.e3.
34. Pallé A, Montero M, Fernández S, Tezanos P, De Las Heras JA, Luskey V *et al.* Gαi2 + vomeronasal neurons govern the initial outcome of an acute social competition. *Sci Rep* 2020; 10: 894.
35. Iezzi D, Caceres-Rodriguez A, Chavis P, Manzoni OJJ. In utero exposure to cannabidiol disrupts select early-life behaviors in a sex-specific manner. *Translational Psychiatry* 2022; 12: 501.
36. Jin C, Kang HR, Kang H, Zhang Y, Lee Y, Kim Y *et al.* Unexpected Compensatory Increase in Shank3 Transcripts in Shank3 Knock-Out Mice Having Partial Deletions of Exons. *Front Mol Neurosci* 2019; 12: 228.
37. Jin C, Kang H, Yoo T, Ryu JR, Yoo Y-E, Ma R *et al.* The Neomycin Resistance Cassette in the Targeted Allele of Shank3B Knock-Out Mice Has Potential Off-Target Effects to Produce an Unusual Shank3

Isoform. *Front Mol Neurosci* 2020; 13: 614435.

38. Pham CTN, MacIvor DM, Hug BA, Heusel JW, Ley TJ. Long-range disruption of gene expression by a selectable marker cassette. *Proceedings of the National Academy of Sciences* 1996; 93: 13090–13095.
39. Meier ID, Bernreuther C, Tilling T, Neidhardt J, Wong YW, Schulze C *et al.* Short DNA sequences inserted for gene targeting can accidentally interfere with off-target gene expression. *The FASEB Journal* 2010; 24: 1714–1724.
40. Hon W-C, McKay GA, Thompson PR, Sweet RM, Yang DSC, Wright GD *et al.* Structure of an Enzyme Required for Aminoglycoside Antibiotic Resistance Reveals Homology to Eukaryotic Protein Kinases. *Cell* 1997; 89: 887–895.
41. Blüthgen N, van Bentum M, Merz B, Kuhl D, Hermey G. Profiling the MAPK/ERK dependent and independent activity regulated transcriptional programs in the murine hippocampus in vivo. *Scientific Reports* 2017; 7: 45101.
42. Iroegbu JD, Ijomone OK, Femi-Akinlosotu OM, Ijomone OM. ERK/MAPK signalling in the developing brain: Perturbations and consequences. *Neuroscience & Biobehavioral Reviews* 2021; 131: 792–805.
43. Coria-Avila GA, Herrera-Covarrubias D, García LI, Toledo R, Hernández ME, Paredes-Ramos P *et al.* Neurobiology of Maternal Behavior in Nonhuman Mammals: Acceptance, Recognition, Motivation, and Rejection. Animals. 2022; 12: 3589.
44. Curley JP, Davidson S, Bateson P, Champagne FA. Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice. *Frontiers in Behavioral Neuroscience* 2009; 3. doi:10.3389/neuro.08.025.2009.
45. Garbugino L, Centofante E, D'Amato FR. Early Social Enrichment Improves Social Motivation and Skills in a Monogenic Mouse Model of Autism, the Oprm1–/– Mouse. *Neural Plasticity* 2016; 2016: 5346161.
46. Gao X, Castro-Gomez S, Grendel J, Graf S, Süsens U, Binkle L *et al.* Arc/Arg3.1 mediates a critical period for spatial learning and hippocampal networks. *Proc Natl Acad Sci U S A* 2018; 115: 12531–12536.
47. Ploski JE, Pierre VJ, Smucny J, Park K, Monsey MS, Overeem KA *et al.* The Activity-Regulated Cytoskeletal-Associated Protein (Arc/Arg3.1) Is Required for Memory Consolidation of Pavlovian Fear Conditioning in the Lateral Amygdala. *J Neurosci* 2008; 28: 12383–12395.
48. Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF *et al.* Inhibition of Activity-Dependent Arc Protein Expression in the Rat Hippocampus Impairs the Maintenance of Long-Term Potentiation and the Consolidation of Long-Term Memory. *J Neurosci* 2000; 20: 3993–4001.
49. Peñagarikano O, Abrahams BS, Herman EI, Winden KD, Gdalyahu A, Dong H *et al.* Absence of CNTNAP2 Leads to Epilepsy, Neuronal Migration Abnormalities, and Core Autism-Related Deficits. *Cell* 2011; 147: 235–246.

50. Raja R, Dumontier E, Phen A, Cloutier J-F. Insertion of a neomycin selection cassette in the locus alters gene expression in the olfactory epithelium leading to region-specific defects in olfactory receptor neuron development. *genesis* 2024; 62: e23594.
51. Hillerer KM, Jacobs VR, Fischer T, Aigner L. The maternal brain: an organ with peripartal plasticity. *Neural Plast* 2014; 2014: 574159.
52. Zheng J, Baimoukhametova D, Lebel C, Bains JS, Kurrasch DM. Hypothalamic vasopressin sex differentiation is observed by embryonic day 15 in mice and is disrupted by the xenoestrogen bisphenol A. *Proc Natl Acad Sci U S A* 2024; 121: e2313207121.
53. Vithayathil J, Pucilowska J, Landreth GE. Chapter 3 - ERK/MAPK signaling and autism spectrum disorders. In: Shekhar ABT-P in BR (ed). *Genetic Models and Molecular Pathways Underlying Autism Spectrum Disorders*. Elsevier, 2018, pp 63–112.
54. Yuan P, Zhou R, Wang Y, Li X, Li J, Chen G *et al.* Altered levels of extracellular signal-regulated kinase signaling proteins in postmortem frontal cortex of individuals with mood disorders and schizophrenia. *Journal of Affective Disorders* 2010; 124: 164–169.
55. Murari K, Abushaibah A, Rho JM, Turner RW, Cheng N. A clinically relevant selective ERK-pathway inhibitor reverses core deficits in a mouse model of autism. *eBioMedicine* 2023; 91: 104565.
56. Dölen G, Malenka RC. The Emerging Role of Nucleus Accumbens Oxytocin in Social Cognition. *Biological Psychiatry* 2014; 76: 354–355.
57. Mohapatra AN, Wagner S. The role of the prefrontal cortex in social interactions of animal models and the implications for autism spectrum disorder. *Frontiers in Psychiatry* 2023; 14. doi:10.3389/fpsyt.2023.1205199.
58. Wei J, Ma L, Ju P, Yang B, Wang Y-X, Chen J. Involvement of Oxytocin Receptor/Erk/MAPK Signaling in the mPFC in Early Life Stress-Induced Autistic-Like Behaviors. *Frontiers in Cell and Developmental Biology* 2020; 8: 564485.
59. Gora C, Dudas A, Vaugrente O, Drobecq L, Pecnard E, Lefort G *et al.* Deciphering autism heterogeneity: a molecular stratification approach in four mouse models. *Translational Psychiatry* 2024; 14: 416.
60. Kim S, Kim H, Um JW. Synapse development organized by neuronal activity-regulated immediate-early genes. *Experimental & Molecular Medicine* 2018; 50: 1–7.
61. Bonsi P, De Jaco A, Fasano L, Gubellini P. Postsynaptic autism spectrum disorder genes and synaptic dysfunction. *Neurobiology of Disease* 2022; 162: 105564.
62. Lin V, Matta SM, Dallman JE, Hosie S, Moreau M, Franks AE *et al.* A Frank Assessment of SHANK: Impacts of Pathogenic Variations in SHANK3 on Preclinical Models of Phelan McDermid Syndrome. *Autism Res* 2025; 18: 1935–1964.
63. Peça J, Feliciano C, Ting JT, Wang W, Wells MF, Venkatraman TN *et al.* Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature* 2011; 472: 437–442.
64. Saré RM, Levine M, Smith CB. Behavioral Phenotype of Fmr1 Knock-Out Mice during Active Phase in an Altered Light/Dark Cycle. *eNeuro* 2016; 3: ENEURO.0035-16.2016.

65. Spencer CM, Alekseyenko O, Serysheva E, Yuva-Paylor LA, Paylor R. Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome. *Genes Brain Behav* 2005; 4: 420–430.
66. Bey AL, Wang X, Yan H, Kim N, Passman RL, Yang Y *et al.* Brain region-specific disruption of Shank3 in mice reveals a dissociation for cortical and striatal circuits in autism-related behaviors. *Transl Psychiatry* 2018; 8: 94.
67. Yang M, Bozdagi O, Scattoni ML, Wöhr M, Roullet FI, Katz AM *et al.* Reduced excitatory neurotransmission and mild autism-relevant phenotypes in adolescent Shank3 null mutant mice. *J Neurosci* 2012; 32: 6525–6541.
68. Brown JR, Ye H, Bronson RT, Dikkes P, Greenberg ME. A Defect in Nurturing in Mice Lacking the Immediate Early Gene fosB. *Cell* 1996; 86: 297–309.
69. Cockayne DA, Muchamuel T, Grimaldi JC, Muller-Steffner H, Randall TD, Lund FE *et al.* Mice Deficient for the Ecto-Nicotinamide Adenine Dinucleotide Glycohydrolase CD38 Exhibit Altered Humoral Immune Responses. *Blood* 1998; 92: 1324–1333.
70. Poliak S, Salomon D, Elhanany H, Sabanay H, Kiernan B, Pevny L *et al.* Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1. *Journal of Cell Biology* 2003; 162: 1149–1160.
71. Meng L, Person RE, Huang W, Zhu PJ, Costa-Mattioli M, Beaudet AL. Truncation of Ube3a-ATS Unsilences Paternal Ube3a and Ameliorates Behavioral Defects in the Angelman Syndrome Mouse Model. *PLOS Genetics* 2013; 9: e1004039.
72. Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K *et al.* Neuroligins Determine Synapse Maturation and Function. *Neuron* 2006; 51: 741–754.
73. Geppert M, Khvotchev M, Krasnoperov V, Goda Y, Missler M, Hammer RE *et al.* Neurexin Iα Is a Major α-Latrotoxin Receptor That Cooperates in α-Latrotoxin Action. *Journal of Biological Chemistry* 1998; 273: 1705–1710.
74. Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, Barrantes I del B *et al.* High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Current Biology* 1998; 8: 1169–1178.

Figures

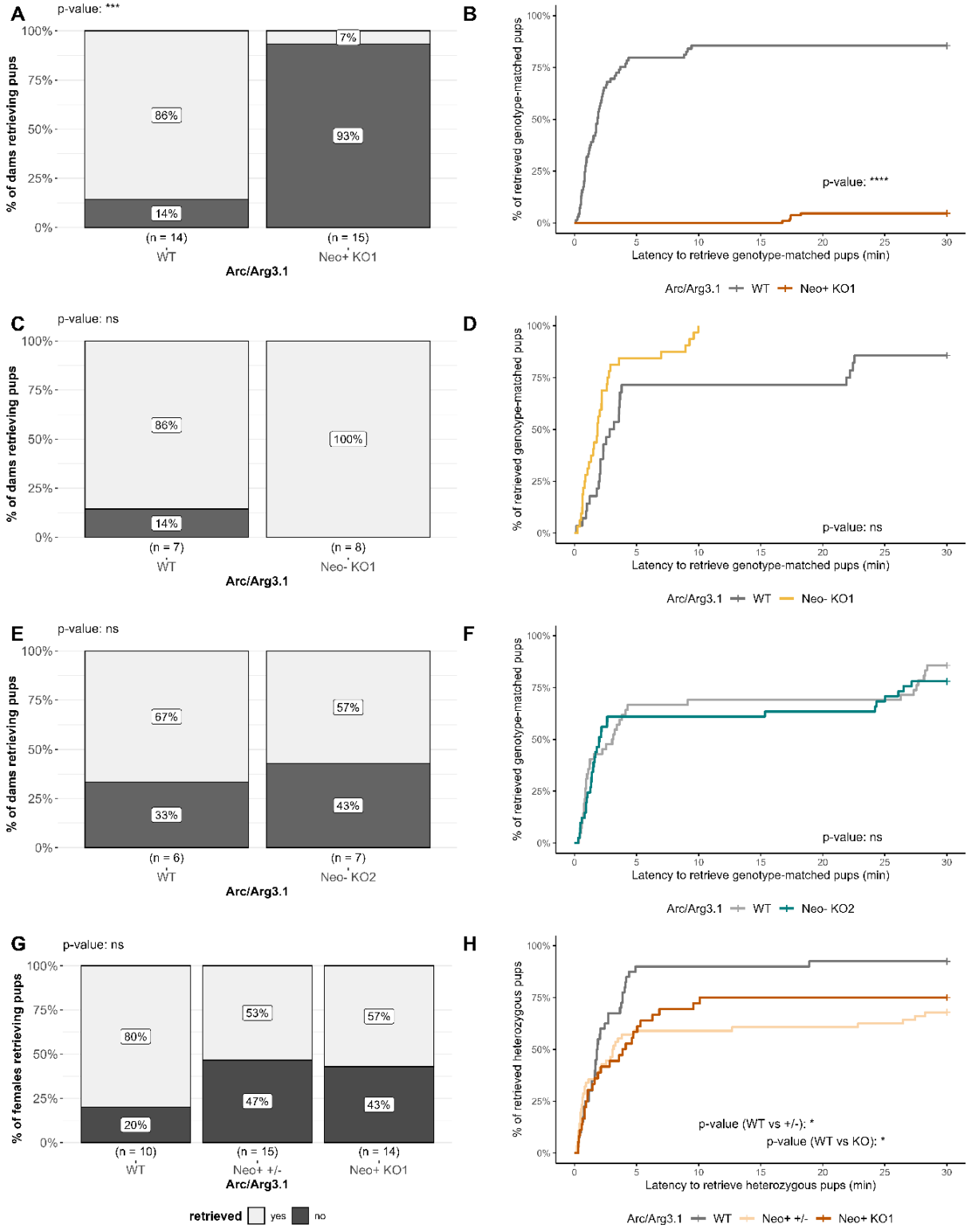


Figure 1

The Neomycin cassette strongly impairs maternal behavior in Neo$^+$ KO dams

In the pup retrieval test, for the first *Arc/Arg3.1* KO1 line raised in mixed C57BL/6J;129S2 genetic background, a higher percentage of Neo$^+$ KO1 dams (brown; n = 15) failed to retrieve their pups compared to WT (dark gray; n = 14) with genotype-matched pups (**A**), resulting in increased pup retrieval

latency (**B**). By contrast, no differences in the percentage of females retrieving their pups (**C**) or pup retrieval latency (**D**) was observed between Neo$^-$ KO1 dams (yellow; n = 8) and WT (dark gray; n = 7). For the second Neo$^-$ *Arc/Arg3.1* KO2 line raised in 100% C57BL/6J genetic background, no difference in the percentage of females retrieving their pups (**E**) or pup retrieval latency (**F**) was observed with their respective WT (pale gray; n = 6), and Neo$^-$ KO2 dams (turquoise; n = 7). The percentage of females retrieving their pups was not different for Neo$^+$ *Arc/Arg3.1*$^{+/-}$ (beige; n = 15) and KO1 dams (brown; n = 14) compared to WT (dark gray; n = 10) with heterozygous Neo$^+$ *Arc/Arg3.1*$^{+/-}$ pups (**G**), with increased pup retrieval latency (**H**). Data are presented as mean ± sd in **Table S1**. Groups in **A**, **C, E**, and **G** were compared by Fisher's exact test, and groups in **B**, **D, F**, and **H** by Kaplan-Meier test, with an asterisk indicating genotype effects. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

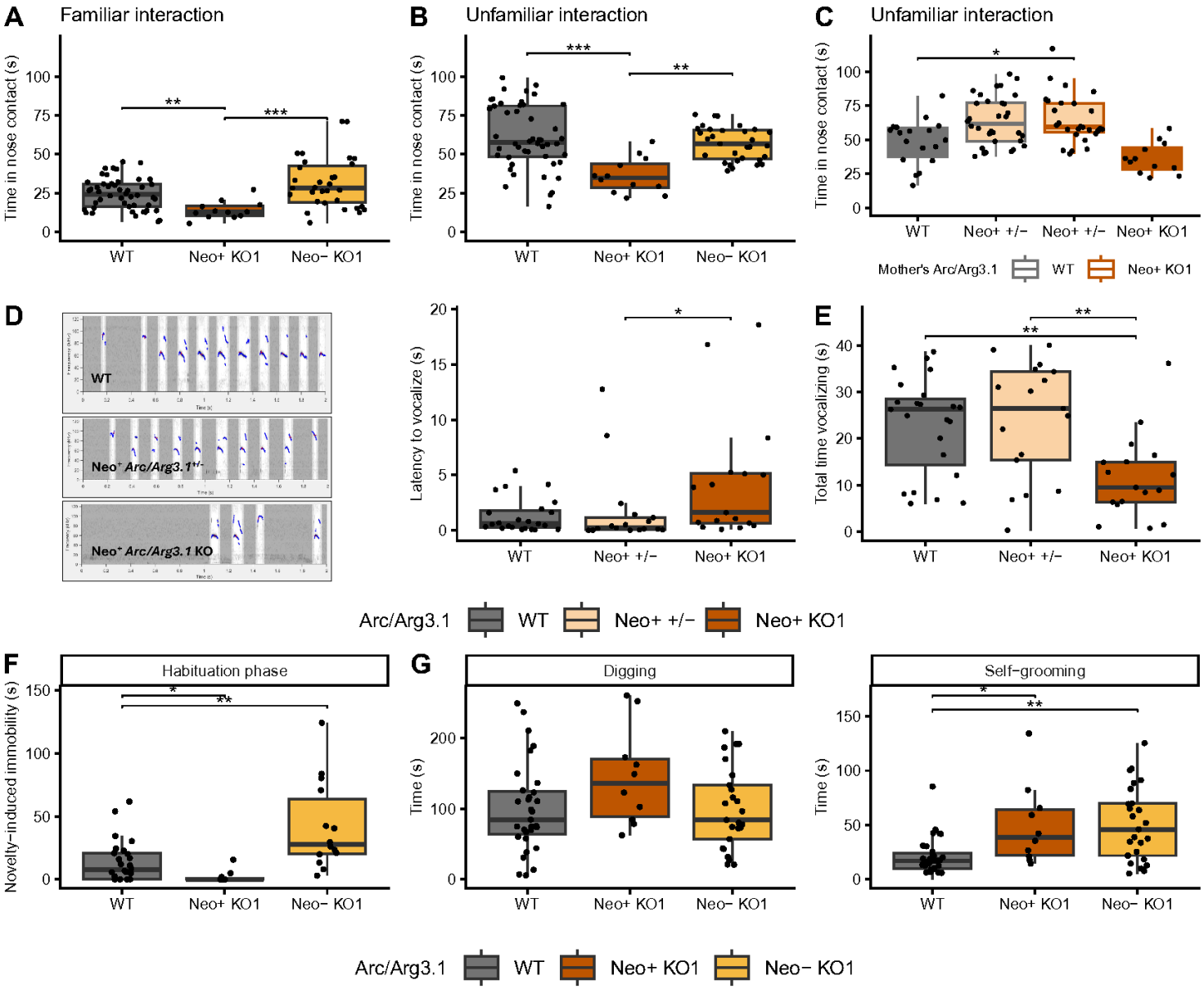


Figure 2

Neo cassette affects social and non-social behaviors in both dams and pups

Neo^+ KO1 mice (brown; n = 12, 6 females and 6 males) displayed social interaction impairments with cage mates (**A**) and unfamiliar conspecifics (**B**) compared to WT (dark gray; n = 48, 23 females and 25 males), which was not the case for Neo^- KO1 mice (yellow; n = 31, 15 females and 16 males). Neo^+ *Arc/Arg3.1*$^{+/-}$ mice raised either by WT (beige with dark gray outline; n = 32, 16 females and 16 males) or Neo^+ *Arc/Arg3.1* KO1 dams (beige with brown outline; n = 27, 15 females and 12 males) displayed no impairments in social interaction with unfamiliar conspecifics compared to WT mice raised by WT dams (dark gray; n = 18, 8 females and 10 males) unlike Neo^+ *Arc/Arg3.1* KO1 mice raised by Neo^+ *Arc/Arg3.1* KO1 dams (brown; n = 12, 6 females and 6 males) (**C**). Upon maternal separation, Neo^+ KO1 pups (brown; n = 17) displayed increased latency to vocalize as shown by representative ultrasound vocalization traces and quantitative data (**D**) and reduced ultrasound vocalizations (**E**) compared to Neo^+ *Arc/Arg3.1*$^{+/-}$ (beige; n = 17) and WT pups (dark gray; n = 24). In the habituation phase of the three-chambered test, unlike Neo^+ KO1 mice (brown; n = 12, 6 females and 6 males), Neo^- KO1 mice (yellow; n = 14, 7 females and 7 males) displayed increased novelty-induced immobility compared to WT (dark gray; n = 26, 12 females and 14 males; **F**). In the motor stereotypy test, both Neo^+ (brown; n = 20, 12 females and 8 males) and Neo^- KO1 mice (yellow; n = 50, 26 females and 24 males) showed increased time self-grooming, but not digging compared to WT (dark gray; n = 62, 30 females and 32 males; **G**). Data are presented as mean ± sd in **Tables S1-3**. All groups were compared by Kruskal-Wallis test followed by Dunn's post-hoc test, with an asterisk indicating genotype effects (p = P adjusted). *p < 0.05; **p < 0.01; ***p < 0.001.

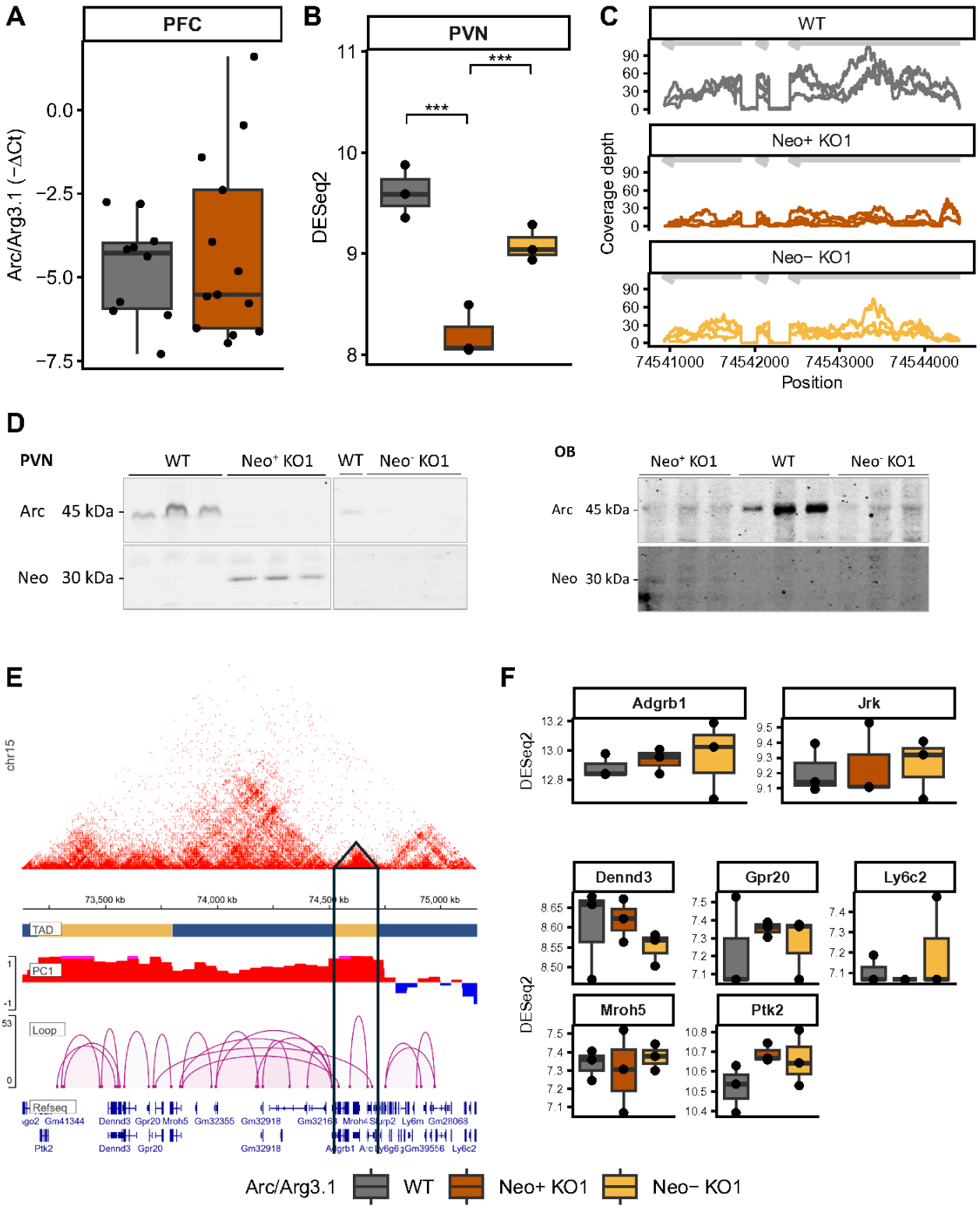


Figure 3

the Neomycin cassette insertion in the *Arc/Arg3.1* locus did not affect Arc protein and neighboring gene expression in the paraventricular nucleus

Arc mRNA levels assessed using qPCR primers targeting the 3'UTR spanning the exon 1-2 region retained in Neo$^+$ KO1 animals (**Figure S1**) were similar in the PFC of WT (dark gray, n = 5 females and 5

males) and Neo^{+} KO1 mice (brown; n = 7 females and 6 males) (**A**). In contrast, RNA-seq analysis revealed reduced Arc mRNA levels in the PVN of Neo^{+} KO1 females, compared with WT or Neo^{-} KO1 (yellow, n = 3 per genotype; **B**). Read mapping showed no evidence of alternative splicing, but indicated an overall reduction in read coverage across Arc/Arg3.1 exons in Neo^{+} and Neo^{-} KO1 mice (**C**). No alternative or fusion Arc protein was detected, while Neo protein expression was observed only in the PVN of Neo^{+} KO1 mice by Western blot (**D**). Analysis of the 3D genomic interaction environment of the murine *Arc/Arg3.1* locus (topologically associated domain on chromosome 15, triangle) suggested potential effect of Neo insertion on nearby gene expression (**E**). In the PVN, genes located at both short (**F**) and long (**G**) distance from the *Arc/Arg3.1* locus expressed similarly to WT levels. Genotypes were compared by the Kruskal-Wallis test followed by Dunn's post-hoc test (A), and DESeq2 (Wald test; B) with an asterisk indicating an effect (p = P adjusted) ***p < 0.001. PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus.

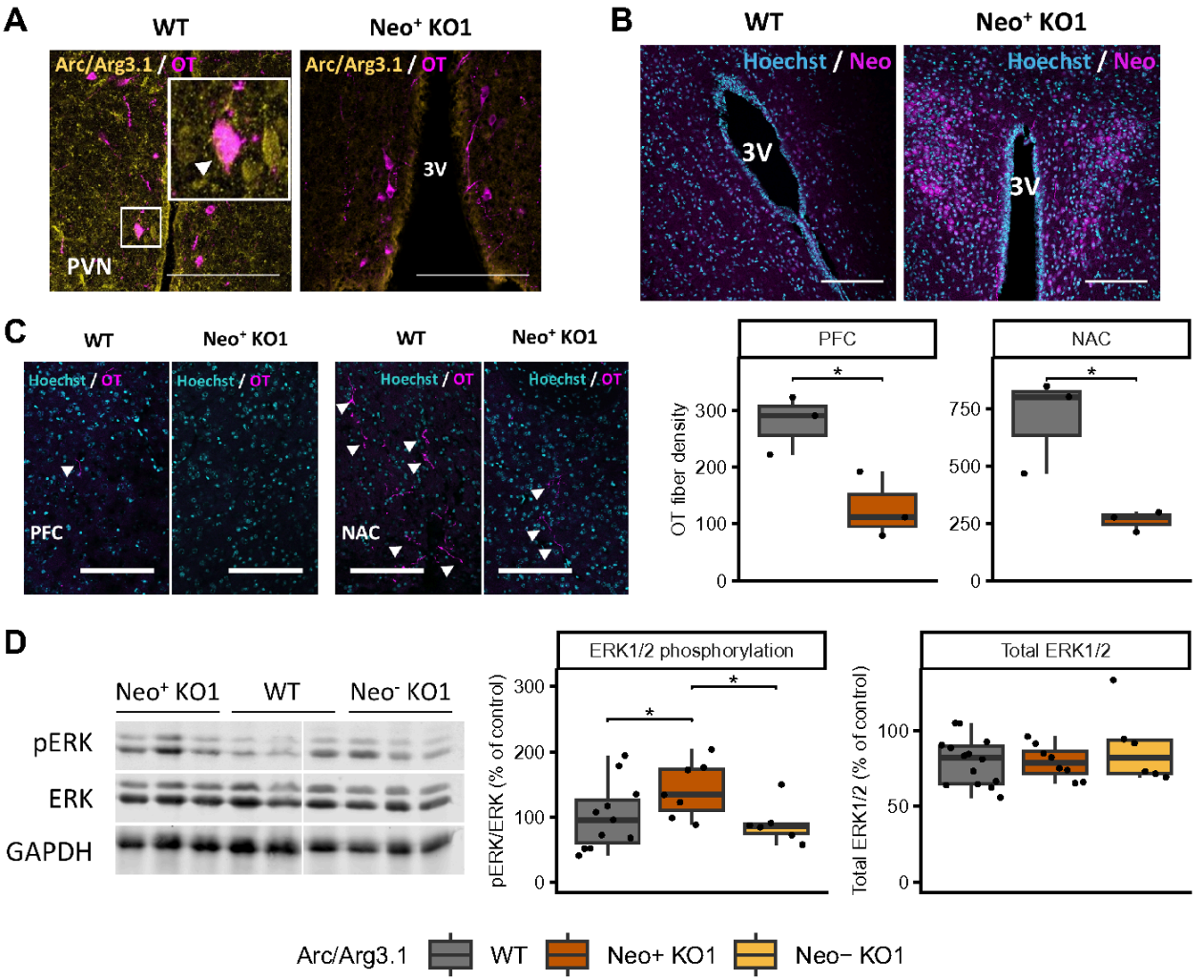


Figure 4

The Neomycin cassette perturbs gene expression, oxytocin neurons and ERK signaling in the PVN

Arc protein is expressed in oxytocinergic (OT) neurons of the PVN in WT, but not in Neo$^+$ KO1 mice (**A**). The Neo product is detected in the PVN of Neo$^+$ KO1, but not of WT dams (**B**). OT neuron fiber density was reduced in the NAC (bregma 0.86) and mPFC (bregma 1.78) of Neo$^+$ KO1 (brown; n = 3) compared

with WT dams (dark gray; n = 3), as shown by representative images and quantification (**C**). Phosphorylated, but not total ERK1/2 in the PVN of Neo^+ KO1 females (brown; n = 7) was increased compared with WT (dark gray; n = 11) and Neo^- KO1 females (yellow; n = 6; **D**). Genotypes were compared by the Kruskal-Wallis test followed by Dunn's post-hoc test, with an asterisk indicating genotype effects (p = P value). Volcano plot and heatmap of differential expression in the PVN of Neo^+ KO1 (brown) *vs.* WT (gray) and Neo^- KO1 (yellow) females using RNA sequencing (n = 3 per genotype; **E**). STRING analysis revealed major reduction in structural components of ribosomes, cell adhesion and, ubiquitin ligase activity (**F**). *p < 0.05; **p < 0.01; ***p < 0.001. 3V, third ventricle; mPFC, medial prefrontal cortex; NAC, nucleus accumbens; Neo product, protein product of the Neo cassette; OT, oxytocin; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; scale bars = 150 μm.

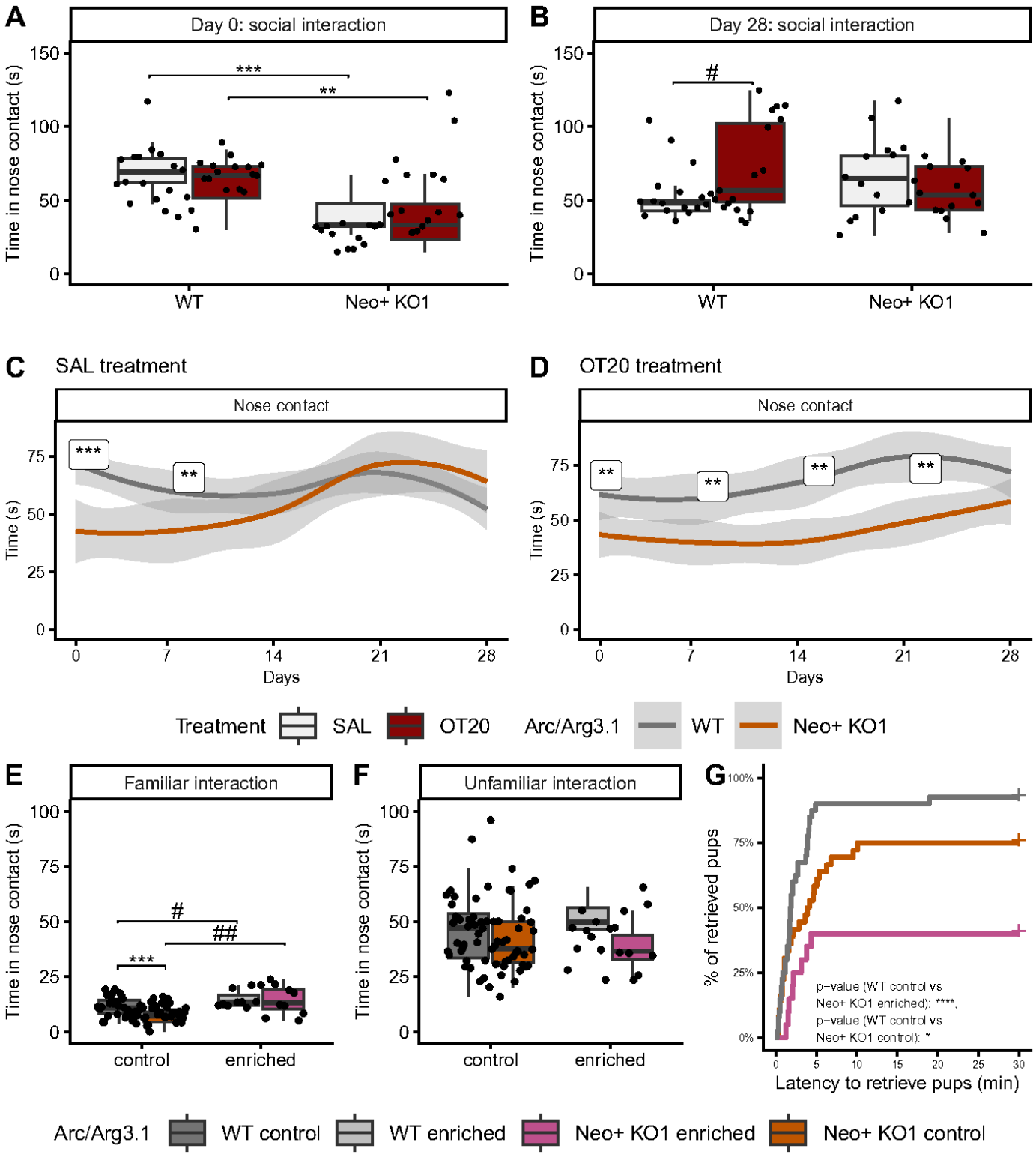


Figure 5

Early social environment enrichment, but not intranasal oxytocin administration partially improves sociability deficits in Neo^{+} KO1 mice

In the LMT, Neo^{+} KO1 mice treated with acute (Day 0) saline (SAL; light gray; n = 8 females and 4 males) or oxytocin (OT) intranasal administration at 20 µg kg^{-1} (red; n = 10 females and 6 males) displayed

reduced social interaction with unfamiliar treatment-matched conspecifics, compared to SAL-treated (light gray; n = 7 females and 8 males) and OT-treated WT controls (red; n = 8 females and 11 males; **A**). OT-treated WT mice displayed increased social interaction compared to SAL-treated WT controls, while Neo^+ KO1 mice show no difference after 28 days of sub-chronic SAL or OT administration (**B**). SAL-treated (**C**) and OT-treated (**C**) Neo^+ KO1 mice showed increased social interaction from day 21 and 28, respectively, while OT-treated, but not SAL-treated WT mice displayed increased social interaction over time. Both Neo^+ KO1 (pink; n = 5 females and 7 males) and WT (light gray; n = 5 females and 2 males) mice raised in early-life social enriched environment with WT and Neo^+ *Arc/Arg3.1*$^{+/-}$ littermates showed increased time in nose contact with cage mates compared to Neo^+ KO1 (brown; n = 12 females and 12 males) and WT mice (dark gray; n = 26 females and 17 males) raised with their sex-, genotype-matched littermates (**E**). However, Neo^+ KO1 mice raised in enriched environment displayed reduced social interaction with unfamiliar conspecifics (**F**). In the pup retrieval test, both Neo^+ KO1 dams raised in the enriched environment (pink; n = 5) and Neo^+ KO1 control (brown; n = 9) displayed increased latency to retrieve Neo^+ *Arc/Arg3.1*$^{+/-}$ pups compared with WT control (dark gray; n = 10; **G**). Data are presented as mean ± sd (**Tables S1 and S2**). Groups in **A-F** were compared by the Kruskal-Wallis test followed by Dunn's post-hoc test and groups in **G** by Kaplan-Meier test, with an asterisk indicating genotype effects and ladder for day or social environment effect (p = P adjusted). # and *p < 0.05; ## and **p < 0.01; ### and ***p < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DudasneoSIR1V4.pdf