Effect of *Esr1* gene polymorphisms on parental behavior in female mice

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CONTENT

ABSTRACT

Parental behaviors are important for survival of offspring. Previous studies implicated that gene polymorphisms play an important role in determining maternal responsiveness, but details of the neural mechanism remain elusive. Estrogen plays an essential role in onset of parental behavior in females (maternal behavior) and exerts its actions through receptors. Estrogen receptor 1 (ESR1) is one of the estrogen receptors (ERs) which is encoded by *Esr1* gene. The expression of ESR1 in hypothalamic medial preoptic area (MPOA) determines the pup retrieval behavior. Here, I addressed how polymorphisms in *Esr1* gene can affect maternal behavior in mice. My study identified that mice from different genetic background, i.e., laboratory strain C57BL/6J (B6) and wild derived strain MSM/Ms (MSM) differ in *Esr1* gene structure. A GCC repeat length polymorphism (9bp) which results in difference of polyalanine repeat numbers was identified in exonic region of *Esr1* gene in MSM when compared to B6. To analyze the role of this "GCC repeat length", we developed an $Esr1^{\Delta 9/\Delta 9}$ mice in which the "9bp" sequence was deleted in B6 mice through CRISPR/Cas9-mediated genome editing. Interestingly, females with heterozygous deletion of "9bp" ($Esr1^{\Delta 9/+}$) showed more severe phenotype than homozygous deletion ($Esr1^{\Delta 9/\Delta 9}$). The low pup (offspring) survival and abnormal maternal behavior (reluctant to retrieve pups) was observed in $Esr1^{\Delta 9/+}$ females. Immuno-histochemical analysis of MPOA region, showed lower number of ESR1 positive cells in $Esr1^{\Delta 9/+}$ females when compared with $Esr1^{+/+}$ and $Esr1^{\Delta 9/\Delta 9}$ females. As MPOA ESR1 positive cells are the key mediators of pup approach and retrieval, abnormal maternal behavior of $Esr1^{\Delta 9/+}$ females can be correlated to lower number of MPOA ESR1 positive cells. $Esr1^{\Delta 9/+}$ females showed higher *Esr1* mRNA expression and lower protein levels when compared to wild type control. Altogether, this is the first study to report how *Esr1* genetic polymorphism affects maternal behavior in mice.

INTRODUCTION

Parental care is critical for offspring's survival. Across mammalian species, females exhibit extremely diverse parental care (maternal behaviors) to ensure the mental and physical well-being of the offspring (*1*). Across species, a wide range of maternal behaviors have been observed, generating questions about how animals recognize their offspring and how brain circuits drive and control maternal display of affection. Previously, several studies shown that endocrine changes during pregnancy and child birth are associated with enhanced maternal care and establishment of new neural circuits. Changes in hormones throughout the pregnancy influence serotonergic and dopaminergic circuits in particular, modify the hormonal receptor levels in brain and thus neural activity (*2*). Most importantly, the increase in hormonal estrogen levels in later phase of pregnancy enhances the induction of maternal care (*3-5*). However, studies on rodents revealed that though virgin mice avoid or attack the pups initially, after a short period of sensitization with foster pups, female mice show nearly spontaneous maternal care (*6, 7*) and rats take several days of pup exposure sessions to accept the pups (*8*). These reports summarize that even though the maternal receptivity depends on several factors, upon pup stimulus, virgin females are also able to retrieve pups.

Maternal behavioral outcomes associated with estrogen depends on its signalling pathways activated by its receptors called estrogen receptors (ERs; ESR1 and ESR2). Among both the receptors, the role of ESR1 in signalling pathways is well studied. Estrogen signalling differs at cellular and molecular level based on whether the signalling cascade initiated by activation of membrane-bound or nuclear ERs. A subpopulation (10–15%) of ERs are membrane-bound and this receptor pool also contributes to the overall estrogenic effect, i.e., they regulate the signalling in non- genomic pathway by interacting with membrane bound proteins (*9*). On the other hand, nuclear ERs facilitate transcriptional

activation in a more classical genomic route, where upon binding with estrogen, ESR1 activated to form a functional dimer (*10, 11*).

The expression of ESR1 in specific brain regions decides the fate of several biological functions and behaviors such as social, sexual, aggression and maternal behaviors (*12-15*). Several reports identified that hypothalamic medial preoptic area (MPOA) is the key region for driving maternal behaviors. The importance of the expression of ESR1 in MPOA for maternal behavior was revealed by several studies. ESR1-KO mice showed reduced estrogen sensitivity and impairment in maternal behavior (*13*), and knockdown of *Esr1* mRNA in the MPOA before pregnancy impaired the emergence of maternal behaviors in lactating female mice (*16*). Furthermore, optogenetic inactivation of MPOA-ESR1 expressing cells impair pup approach and retrieval (*14*). Even though, maternal neural circuits are well studied throughout the years, it is not yet clear which estrogen signalling pathway controls the parental behavior outcome.

The single nucleotide polymorphisms (SNPs) in *Esr1* gene are widely studied in human clinical studies. It was known that this gene polymorphisms directly or indirectly lead to variation in its activity. The two major SNPs, PvuII (rs2234693) and XbaI (rs9340799) are named after their cleaving sites of restriction enzymes. Initially these polymorphisms are known to be associated with loss of bone mineral density (*17*) and breast cancer (*18*). Later, many studies reported their role in altered behavioral phenotypes, like aggression (*19*), episodic memory (*20, 21*), anxiety (*22*), depression (*23*), obsessive-compulsive disorder (*24*) and harsh maternal parenting (*25*). Still, due to inconsistency of findings across the studies, the defined neural circuits which regulate the change in the behavior are uncertain.

The studies on role of *Esr1* polymorphisms in other species are sparse. There is only one study on white-throated sparrows where the chromosomal segregation resulted in SNPs

in promoter region of *Esr1* gene and also behavioral phenotypes. This polymorphism has effect on *Esr1* mRNA expression levels i.e., higher or lower specific to the brain region and also lead to overt social and parental behavior (*26*). Mice are widely used model species for studying molecular and neural mechanism underlying maternal behavior, none of the studies were focussed on effect of polymorphisms.

In the present study, *Esr1* gene sequence of 18 mice strains (Table 1; http://www.informatics.jax.org/marker/MGI:1352467) was compared to check whether there is any variation in the gene sequence. Specific primers were designed to identify the polymorphism in the coding region of interest. We used one of the wild derived mice strains MSM/Ms (MSM; found in Mishima, Japan) and commonly used laboratory strain C57BL/6J (B6) in this study. These mice strains are already reported to have a difference in their behavior, where MSM shows higher aggression and social behavior when compared to B6 (*27, 28*). The coding exon 1 sequence of *Esr1* gene of MSM was compared with B6 by performing the sequence analysis. A 9bp (GCC; codes for alanine; AAA) coding polymorphism was identified in B6 (Figure 2). This 9bp is present in the long stretch of GCC (alanine) repeat region and this kind of trinucleotide repeats are common in human and rodent genome. The importance of presence of alanine repeats in ESR1 are not studied yet, however in a more general way, these repeats are known to be causative for several congenital diseases with neurocognitive phenotypes in humans. It is therefore of general interest to explore the significance of this 9bp polymorphism in B6 genome. In the present study, I aimed to explore the importance of 9bp presence in B6 *Esr1* gene in the context to maternal behavior.

METHODS

Esr1 gene sequence alignment

To select the region for identification of polymorphism, I downloaded the full- length *Esr1* gene sequences of 18 different wild and inbred mice strains (Table 1) from MGI (mouse genome informatics; http://www.informatics.jax.org/). Only coding exons were considered for alignment. Nucleotide and translated protein sequences were aligned in MEGA11 using the MUSCLE algorithm followed by visual screening for polymorphisms.

Study animals and polymorphism identification

I used two mouse strains for polymorphism identification. The first one is wild-derived mouse strain MSM/Ms (MSM). MSM was established as an inbred strain at National Institute of Genetics after inbreeding for more than 20 generations (*29*). The second one is commonly used laboratory mouse strain C57BL/6JJcl (B6), purchased from CLEA Japan, Inc. These mice have been kept in NIG animal facility. Based on the results of *Esr1* gene sequence alignment (mentioned above), I designed specific PCR- primers for coding exon 1 to check whether MSM has any sequence variability in comparison with B6. The genomic DNA of both MSM and B6 was subjected to genomic PCR using a KOD DNA polymerase (TOYOBO global) and primer set (Invitrogen; forward: AACAGCAAGCCCACTGTGTTC, reverse: GGCTCGTTCTCCAGGTAGTAGG). These PCR products were sequenced (https://eurofinsgenomics.jp/jp/service/dnasequence/order-start/) and aligned to identify the *Esr1* gene sequence variation.

Animals

All animal procedures were performed in accordance with the relevant guidelines and regulations approved by the Institutional Animal Care and Use Committee of the NIG,

Japan. *Esr1*$^{\Delta 9/+}$ mice were generated in B6 background and crossbred each other to generate littermate pairs of *Esr1*$^{+/+}$, *Esr1*$^{\Delta 9/+}$, and *Esr1*$^{\Delta 9/\Delta 9}$. Progeny were weaned at 21–28 days of age and housed in same-sex groups of 3–5 animals and randomly selected for behavioral and molecular experiments. The breeding and maintenance of all mice were done under specific-pathogen-free (SPF) conditions. The mice were housed in a temperature-controlled room at 23 ± 2 °C following a 12 h light and 12 h dark cycle and provided food and water *ad libitum.* Test mice used for maternal behavior and molecular experiments were all adult female (age: 12-17 weeks). Pups (post-natal; P1-3) for the maternal behavior experiment were obtained by breeding the Jcl: ICR mice (CLEA Japan). All the experiments were performed during the light cycle of the animals.

Generation of Esr1*$^{\Delta 9/\Delta 9}$ *mice

Genome editing to delete 9bp in *Esr1* gene was conducted using the CRISPR/Cas9 nuclease system. The CRISPOR (*30*) and CRISPR Guide RNA Design (https://www.benchling.com/crispr/) were used to design specific and guide RNA sequences. Template DNAs for *in vitro* transcription of the guide RNAs were PCR amplified by Tks Gflex DNA Polymerase (TaKaRa Bio) with a primer set containing the sequences of the T7 promoter GGATCCTAATACGACTC ACTATAGGG and the target site GTAGACCGGCGCCGAGG CGGCG G. The PCR products were purified by Fast Gene Gel/ PCR Extraction Kit (NIPPON Genetics). The guide RNAs were synthesized with the MEGA shortscript™ T7 Transcription kit (Thermo). The synthesized guide RNA product was purified using a MEGAclear™ Transcription Clean-Up kit (Thermo). The guide RNA (50 ng/µl) and CAS9 protein (Integrated DNA Technologies; 50 ng/µl) were microinjected into the pronuclei of fertilized B6 eggs. The injected embryos were transferred into the oviduct of pseudo pregnant female ICR mice. Genotyping of the

progeny was conducted by genomic PCR to detect 9bp deletion. A correctly targeted allele was confirmed by direct sequencing of the PCR product. Generation of *Esr1*$^{\Delta 9/\Delta 9}$ mice was performed by Mr. Yuji Imai and Dr. Akira Tanave.

Breeding records

To quantify and compare breeding potential of females, *Esr1*$^{+/+}$ (wild type), *Esr1*$^{\Delta 9/+}$ (heterozygotes), *Esr1*$^{\Delta 9/\Delta 9}$ (homozygotes) females were crossed with B6 males. Breeding records were maintained for several generations and total number of litters obtained during the given period (100 days) was noted. At least 8 breeding females were taken into account to count the pup survival. Pup number was counted on the postnatal day 1 (P1, followed by next 5 days. In the end, number was recorded on the weaning P25. Pup survival rate was calculated by number of pups born and weaned (in each litter). Shortly after weaning, mice ear tissue was taken by punching to perform PCR-based genotyping.

Maternal behavioral analysis

Virgin female mice (age: 9-17 weeks, n=36, 12 for each genotype) were used for performing maternal behavior. The protocol for performing maternal behavior was partially obtained from the earlier report (*31*). Females were individually habituated for 2 days for experimental home cage before performing behavioral analysis (Figure. 5A). Three donor ICR pups (age: 2-3 days) were placed in separate corners of each test cage over 10-minute period and time taken for retrieving each pup was measured. This process was repeated for 5 times with a maximum 2-minute interval (Figure. 5B) and in every session, we used new pups (15 pups in total). On whole, total exposure time was about 50 minutes and it is referred as initial exposure (IE) session. After this session pups were

removed and mice were left alone in the cages. Four days after the IE, the retrieving test was conducted again only for one session. This is referred as re-exposure session (RE). The time taken for approaching and retrieving three pups were calculated for each pup separately in both IE and RE sessions. The total time females spent with all the three pups performing various maternal gestures like pup licking, hovering and nursing was calculated.

Video acquisition and behavioral analysis

Maternal behavior experiments were video recorded from the top of the cage using a digital video camera (Sony; Figure. 5C) and analysed in a manual behavioral annotation. The following parameters were considered for analysing the maternal behavior based on a previous report (*14, 32*). First, pup approach and retrieval were calculated as the time taken by female mice to approach and retrieve all the three pups separately. Next, pup-licking was calculated as a time taken by female to lick the pups at least continuously for 5 seconds timeline in which the female licked a pup or pups two times or more. Pup nursing behavior includes when the female mice maintain a close contact with the pups by licking or even staying close to pups. Hover refers to the upright nursing position of female over pups throughout the 10-second interval with hind legs in a fixed position. Time spent by female performing pup licking, nursing and hover was combined for all the three pups.

Perfusion fixation and Nissl staining

Adult females were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After the fixation, the brains were dissected out and post-fixed for overnight in 4% PFA and cryoprotected in 30%

sucrose solution for next 24 hours. Brains were embedded in Shandon M-1 embedding matrix (Fisher 1310) and coronally sliced on cryo-microtome into 30µm thick sections. After cutting, sections were washed with PBS-T (0.3%Triton X) for at least 5 times (5x5) and pasted on a glass slide in accordance with the mouse brain stereotaxic coordinates and allowed to air dry. For Nissl staining the sections were dehydrated with alcohol and rehydrated in 0.1 % Toludine blue solution (Sigma) for 10 minutes. After rinsing, sections were dehydrated and mounted with mounting medium (Merck). Microscopic observation for identification of brain areas was done using BX-7100 analyser.

Immunostaining

ESR1 and cFOS immunostaining was performed following free-floating method. For the cFOS analysis, all the experimental females (n=4 of each) were perfused using the same procedure mentioned above 90 minutes after the pup exposure. Females were exposed to pups for 30-minute time period in order to detect the cFOS immunoreactive cells. 30-minutes was considered as optimal for detecting the cFOS protein expression after the pup exposure (*33*). For immunostaining , brain sections were collected in PBS, washed in PBST (3×5 minutes), blocked in 1% normal donkey serum (NDS) and incubated for 30 minutes at room temperature (RT) in a shaker. For double immunostaining of ESR1 (rabbit polyclonal antibody; Millipore) and cFOS (rabbit monoclonal antibody; Abcam) were used. I used step wise protocol as both the antibodies were raised in rabbit. After blocking, sections were incubated overnight with cFOS primary antibody and followed by incubation with goat anti-rabbit IgG Fab recombinant secondary antibody (Alexa Fluor™ 488; Invitrogen). After 1 hour incubation sections were washed thoroughly with PBST (3 × 5 minutes) and blocked again with 1% NDS. Then, sections were incubated overnight with ESR1 antibody. For detecting ESR1, I used IgG (H+L) goat anti-rabbit (Alexa Fluor™ 594; Invitrogen)

secondary antibody. After secondary antibody, sections were washed with PBST (3×5 minutes) and incubated with Neuro Trace 435/455 (blue fluorescent Nissl stain life technologies, 1:200) for 2 h at RT, washed with PBST (3×10 minutes) and mounted on slides and cover slipped with mounting medium (Invitrogen).

Image analysis

The 4× or 10× magnification fluorescent images were acquired to determine the overall protein expression pattern. For counting ESR1 and cFOS positive cells 20× fluorescent confocal images were acquired. Positive cells were automatically counted by using Image J quantification plugin colocalization object counter. Different colour filters (ESR1: red, cFOS: cyan, ESR1 +cFOS: white) were used to detect the expression of each protein separately. The setting (noise tolerance) was maintained to avoid the detection of noise. For each brain, all the images were tried to take at same position and settings.

Estrus cycle monitoring and plasma Estradiol levels

For checking the estrus stage of the female mice we followed vaginal swab method. Briefly, from the vaginal wall of restrained mouse, a smear was collected using a wet cotton swab (dipped in saline) and cells were transferred to a dry glass slide. The slide was air dried and then stained with approximately 100 µL of Giemsa stain (Wako) for 1minute. The slides were rinsed with water, and viewed immediately at 20× magnification under bright field microscope (Olympus). The stage of the estrus cycle was determined based on the presence or absence of leukocytes, cornified epithelial, and nucleated epithelial cells (*34*). At proestrus stage by cardiac puncture blood was collected and immediately plasma was isolated and stored till the assay. Estradiol concentration was measured in technical duplicates (n=6) following manufacturer's instructions (Cayman ELISA Estradiol kit).

RNA isolation and qRT-PCR

Hypothalamus samples were collected when female is in proestrus stage. These samples were homogenized on ice in Trizol (Invitrogen). Total RNA was extracted and the quantity and quality were checked using a spectrophotometer (NanoDrop). After DNase treatment (TURBO DNA-free™ kit; Ambion), cDNA was synthesized from hypothalamus using primescript reverse transcriptase (TaKaRa Bio). All cDNA samples were stored at -20°C until qRT-PCR. The expression level of *Esr1* mRNA transcript was quantified in a Thermal Cycler Dice® Real Time System (TaKaRa Bio) using SYBR Premix Ex Taq II (Perfect Real Time; TaKaRa Bio) and primer set; *Esr1* F_ATGATGAAAGGCGGCATACG and *Esr1* R_ TTCGGCCTTCCAAGTCATCT. All reactions were run in technical duplicates, and comparative CT method ($2^{-\Delta\Delta}$CT) method was used for quantifying the expression level of mRNA.

Western blotting

Total protein from hypothalamus collected from the selected female mice sacrificed at 12-15 week (n=6 each) was extracted upon homogenization with 2x Laemmli sample buffer (Bio-Rad). The lysed samples were heated at 95°C for 5 min and subjected to electrophoresis on 4-12 % SDS-polyacrylamide gel (Bio-Rad). The gels were transferred onto PVDF membrane using semi-dry method (Bio-Rad) and were blocked with PVDF blocking buffer (TOYOBO) for 1 h at RT. The blocked membranes were incubated with one of the following rabbit (polyclonal) and mouse (monoclonal) primary antibodies overnight at 4 °C: ESR1 (Millipore 1:10000) and GAPDH (ORIGENE 1:10000). The membranes were then washed with TBS-T and incubated with goat anti-rabbit or anti- mouse HRP-conjugated secondary antibody (Millipore: 1:10000) in TBS-T for 1 h at RT. After washing with TBS-T, chemiluminescence immunoreactivity was detected by using clarityTM western ECL

substrate (Bio-Rad). To control protein loading on the gels, the membranes were stripped, blocked and further probed with the GAPDH antibody. Membranes were developed in high resolution, and digital images were exported to ImageJ for the expression level check. The detailed quantification of expression was explained in figure 11.

Statistical Analysis

The results were presented as mean ± standard deviation (S.D). The statistical analyses were performed by ANOVA. Normality and homogeneity of variances were checked and confirmed to match with parametric assumptions prior to running ANOVA. Significant differences between the means were determined by analysing the data using the Tukey honest significance difference (Tukey HSD) test. The level of significance was set at *P< 0.05. All the graphs and statistical analysis was done by using graphpad prism v 9.3.1. Error bars represent ± SD.

RESULTS

Sequence variation within the Esr1 coding region

The *Esr1* gene sequences of 14 inbred and 4 wild-derived mouse strains were retrieved from MGI database (Table 1). I focused on identifying the variation with in the coding region. For this purpose, I extracted the B6- *Esr1* coding exon sequence from ensemble database (*Esr1* gene contains 8 coding exons). All the 18 strains' *Esr1* gene sequences were aligned with the coding region, to identify the coding region in these strains. Once the coding region of all the mouse strains was retrieved from whole gene, these nucleotide sequences were aligned in MEGA11 using MUSCLE algorithm to identify different polymorphisms in the coding region. More than 20 SNPs were identified; however, these polymorphisms are random among wild and laboratory strains. The length of GCC repeat region in exon 1 was distinctly different between and wild and laboratory strains.

Variation in GCC repeat sequence of Esr1-exon 1 between inbred and wild derived strains

In 13 out of 14 inbred strains, a 9-trinucleotide repeat (TNR) of GCC (27bp) was found and the repeat length was confined to 8 (24bp) in case of wild-derived strains (Figure 1A). Interestingly, an obese model mice NZO/HILtJ consists only 6 repeats (18bp) unlike other mouse strains. From the ensemble database, this was identified as an in-frame deletion (GCCGCCGCC- 9bp)- polymorphism rs245996394 (http://asia.ensembl.org/Mus musculus/ ENSMUSG00000019768). TNRs are highly frequent in human and rodent genomes; these repeats have known to modulate many different functions based on their location and length. For example, the increase in length of a well-studied TNR; CAG in *AR* gene (Androgen Receptor) causes spinal and bulbar muscular atrophy (*35*) and in the *HTT* gene it results in Huntington's disease (*36*). Similarly, one more TNR; CGG repeat length variation in the *FMR1* gene (Fragile X mental retardation) is known to be an underlying cause of fragile X

syndrome (FXS) and also mental retardation (*37*, *38*). Although, the role of GCC repeats was not established, based on the common knowledge of TNRs importance in several neurological diseases, I decided to identify the *Esr1* gene polymorphism in this region.

Next, I proceeded to check the variation in translated protein sequences (Figure 1B). GCC codes for amino acid alanine (A) and this region belongs to the N-terminal (AF1) domain of the ESR1 protein. This domain plays an active role in receptor-dependent transcription and also modulating the interactions with ligand binding domain (LBD) (*39*). These polyalanine regions in transcription factor genes reported to be associated with congenital diseases in human (*40*). Many polyalanine alleles give rise to less severe or disease phenotypes, consistent with partial or complete loss -of protein function (*41*). Based on sequence alignment results of already available mouse strains, the variation in ESR1 protein between inbred and wild-derived mice lead me to look further into this region. Thus, in the present study I focussed on identifying whether this GCC repeat region is varied between one of the wild-derived strain MSM in comparison with commonly used B6 mice.

Esr1 gene variant rs245996394 is identified in B6 in comparison with MSM

In order to identify the variation in GCC repeat of exon 1 between MSM and B6, primers were designed to amplify this region. The genomic DNA was subjected to PCR, followed by sequencing. The sequence analysis showed that GCC repeat length was 18bp in MSM missing the 9bp similar to NZO/HILtJ strain (Figure 2C). As mentioned earlier, this region is in an alanine rich region of AF1 domain which mediates transcriptional activity. One of the splicing variants of *Esr1* (without AF1 domain) forms a protein product at 46kD, where ESR1 canonical protein appears at 65kD (Figure 2D). The specific function of AF1 domain in ESR1 protein is not well-known. Earlier, few reports described that poly-A tract expansions have role in several human diseases or malformations (*42*). Hence, I also

analysed the human *ESR1* gene and protein (sequence obtained from NCBI: NC_000006.12) and identified that 12bp (9+3bp) is absent which is partially aligning with MSM (Figure 2B & C). This suggests that GCC repeat sequence length is highly varied among the species. Despite the variation in sequence length, it is clear that functional ESR1 protein was present in both B6 and MSM irrespective of the presence or absence of 9bp.

As trinucleotide repeats were known for their instability across the genome, I assumed that GCC repeat in *Esr1* gene may not affect the function of protein directly in each species. Still, based on present sequence alignment results which indicates that it is important to explore the implication of variation in GCC repeat (9bp polymorphism) between wild and inbred strains. Thus, I decided to identify the significance by deletion of 9bp in B6 background using CRISPR technology.

Esr1$^{\Delta 9/+}$ female's offspring (pups) showed reduced survival

Using the CRISPR/Cas9 nuclease system, 9bp in the *Esr1* gene was deleted in B6 mice (Figure 3A). When the *Esr1*$^{\Delta 9/+}$ mice were obtained, they were cross bred to generate littermate pairs of *Esr1*$^{+/+}$, *Esr1*$^{\Delta 9/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$. The genomic DNA of *Esr1*$^{\Delta 9/\Delta 9}$ mice was subjected to amplification and followed by sequencing to confirm the 9bp deletion (Figure 3B). In initial breeding records, I observed a lower offspring survival in some of the *Esr1*$^{\Delta 9/+}$ females. This observation led me to check the breeding efficiency of these females. To quantify and compare breeding efficiency of females, *Esr1*$^{+/+}$, *Esr1*$^{\Delta 9/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$ females were crossed with B6 males. *Esr1*$^{\Delta 9/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$ mice showed normal reproductive potential (i.e., number of pups born) when compared to *Esr1*$^{+/+}$ females (Figure 4C). However, higher mortality was observed in the offspring obtained from *Esr1*$^{\Delta 9/+}$ females (Figure 4A and B). Number of litters obtained by each female and number of pups survived based on their percentage was plotted. At least 3-4 *Esr1*$^{\Delta 9/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$ females

(n=12) did not produce any or less litter even after multiple mating trails. A detailed pup survival statistics in each litter was shown in figure 4B (B6 vs *Esr1* $^{\Delta 9/+}$ - P= 0.002; B6 vs *Esr1* $^{\Delta 9/\Delta 9-}$ P=0.865; *Esr1* $^{\Delta 9/+}$vs *Esr1* $^{\Delta 9/\Delta 9}$ - P = 0.029). The number of pups born and weaned by each female was shown in figure 4C. The number of pups born by each female was similar irrespective of genotype, but the number of pups weaned decreased significantly in *Esr1*$^{\Delta 9/+}$ females (B6 vs *Esr1* $^{\Delta 9/+}$ - P= 0.001). Surprisingly the breeding of *Esr1*$^{\Delta 9/\Delta 9}$ females was better when compared to *Esr1*$^{\Delta 9/+}$ females. I did not observe any sex effect on the pup survival (Figure 4D).

At the time of birth, all the pups appeared healthy irrespective of their genotype. To check whether the genotype of the pup has any effect on the survival I considered *Esr1*$^{\Delta 9/+}$ breeding where three types of genotypes can be obtained (Figure 4E). I observed that all the three genotypes are able to survive, eliminating the probability of lethality with respect to genotype of the pups. I performed chi-square test to find out whether there is any difference in the distribution of the three genotypes. I did not find any difference in the distribution ($P>0.05$). indicating that there was no defect in the offspring with respect to genotype. The survived/weaned pups showed no difference in the body weight growing up, at different age groups. They seem healthy and are able to produce progeny after the mating. Taking together, I concluded that the genetic differences of the pups did not cause the loss of survival.

These pup survival results suggest that there were no apparent abnormalities associated with *Esr1*$^{\Delta 9/+}$ females. In addition, I observed the pup mortality within 10 days of their birth and also there was no indication of milk in their stomach (home cage observation). These observations led me to check whether *Esr1*$^{\Delta 9/+}$ females are able to care for their pups or not.

Esr1$^{\Delta 9/+}$ virgin females showed abnormal pup retrieving behavior

To analyse whether the significant reduction in survival of pups with *Esr1*$^{\Delta 9/+}$ females is related to lack of maternal care, I performed maternal behavior analysis of virgin females (Figure 5A). Virgin wild-type female mice were widely used in testing maternal behaviors as they spontaneously exhibit full maternal behaviors when exposed to foster mouse pups. Moreover, hormonal changes caused after pregnancy and post-partum can be avoided with virgin females. Pup exposure assays were performed on five consecutive sessions on days 3 and 8. During the testing, three pups were introduced in the three different corners of female's home cage, and female and pup interaction was observed for 10 min (Figure 5B-C). Different pups were used in each session. I observed that *Esr1*$^{\Delta 9/+}$ females displayed impaired maternal care (Figure 5D). The average latency to approach and retrieve all three pups was longer with *Esr1*$^{\Delta 9/+}$ mice (Figure 5E). Comprehensively, in the initial sessions females of all three genotypes hesitated to approach the pups, but in later sessions they are quick in approaching and sniffing the pups (~ 5sec) (Figure 5E). To approach 1st and 2nd pups' females took almost similar time irrespective of the genotype. However, in case of 3rd pup approach, *Esr1*$^{\Delta 9/+}$ females took longer time in approaching (Figure 5E; Initial exposure- P=0.031 and re-exposure- P=0.043). The average latency to retrieve the first pup was less than 30 s in the control group, and all pups were retrieved within the 10-min testing period (Figure 5F). In case of *Esr1*$^{\Delta 9/\Delta 9}$ even though the average latency increased when compared to *Esr1*$^{+/+}$ females, but not statistically different (2nd pup; P=0.564: 3rd pup; P=0.835). However, *Esr1*$^{\Delta 9/+}$ females took longer time even after repeated pup exposure sessions (Figure 5F; 2nd pup; P=0.014: 3rd pup; P=0.013). Around 5/12 females often ignored the pups and did not show any interest in contacting the pups for the majority of time during the session. However, as the session progresses, I observed more of a contacting behavior in rest of the females (Figure 6).

Other pup-directed behaviors like nursing (time spent by female in contact with the pups) were not significantly changed between the genotypes. However, the other measurements like crouching over and pup licking which involves active females' involvement with pups was observed to be lower in *Esr1*$^{\Delta 9/+}$ females (Re-exposure session- *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$ -nursing; P=0.135: Pup licking; P=0.002: crouching; P=0.001). I observed a significant difference in behaviors during the re-exposure sessions between the genotypes (Figure 6A-C). Overall, these results indicate that *Esr1*$^{\Delta 9/+}$ females were less observant towards the pups and maintained lower contact which might have resulted in lower offspring survival in these females.

Heterozygous deletion of 9bp led to reduced expression of ESR1 in MPOA

The abnormal maternal behavior displayed by *Esr1*$^{\Delta 9/+}$ females led me to check whether 9bp deletion has any impact on ESR1 expression. For this purpose, I first examined the expression of ESR1 in hypothalamic MPOA. This brain region is considered one of the critical brain areas to determine maternal behaviors and also ESR1 expression in MPOA is important for pup retrieving behavior. I also checked the expression of one of the neuronal activation markers, cFOS, which is generally used as an indicator for neuronal activation after stimulation. Previous studies showed a high expression of cFOS neurons in the MPOA region of females after pup exposure (*6, 33, 43*). Thus, I analysed the expression of both ESR1 and cFOS positive cells in MPOA.

To reveal how ESR1 and cFOS protein expression changed in MPOA, immunostaining was performed in 4% paraformaldehyde fixated brains. I considered a bregma range correspond to MPOA according to mouse brain atlas (Figure 7A). In hypothalamus, the preoptic area occupies the bregma range 0.50 to – 0.58; the medial area

of MPOA 0.14, -0.10, -0.22 and -0.34 was used for analysis in this study. Brain sections were divided into two halves and performed the staining. As the distribution of ESR1 in MPOA is also depends on the area, initially, immunostaining using IHC-DAB method was performed (Figure 7A). I confirmed the distribution of ESR1 positive cells was similar to previous reports (*13, 14*). Next, double immunostaining of ESR1 and cFOS was conducted after 30 -minute pup exposure (Figure 7B) to observe the expression of ESR1 and also to check whether MPOA ESR1 positive cells are excited upon pup exposure.

First, ESR1 positive cells were counted in all three genotypes. *Esr1*$^{\Delta 9/+}$ females showed lower ESR1 positive cells when compared to *Esr1*$^{+/+}$ females (Figure 7D). The automatic counting of cells was done by using the plug-in in ImageJ- colocalization object counter, by setting up the red filter for the analysis. The graph was plotted by calculating the percentage of ESR1 positive cells per Neuro Trace. During this process I noted that many of the ESR1 positive cells in *Esr1*$^{\Delta 9/+}$ showed weak expression signals. To look further into the weak ESR1 expression in *Esr1*$^{\Delta 9/+}$ females, ROI (region of interest) method was used to quantify the intensity of each cell in the high magnification (Figure 8A). Briefly, a circle was drawn around each ESR1 positive cell body and intensity of each cell was measured. After the intensity measurement, the cells were categorized into strong and weak expression based on the intensity cut-off. The number of weakly expressing ESR1 positive cells in *Esr1*$^{\Delta 9/+}$ is higher when compared with *Esr1*$^{+/+}$ females (Figure 8). These results suggest that heterozygous deletion of 9bp in B6 genetic background reduced the expression of the ESR1 protein, but the homozygous deletion background did not.

Lower cFOS protein expressing cells in Esr1*$^{\Delta 9/+}$ *females

cFOS immunoreactivity during pup exposure can be analysed by expression analysis. I found no variation in cFOS expression levels when the females were alone (Figure 9A;

P=0.127), indicating that without any external stimulation (like in basal state), cFOS immunoreactivity was similar in all three genotypes. However, upon pup exposure *Esr1*$^{\Delta 9/+}$ females showed lower cFOS expressing cells when compared to the control group (Figure 9B and C; *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.012). As *Esr1*$^{\Delta 9/+}$ females showed less interaction with pups during the pup exposure, cFOS expression is not increased in these females. Next, I also checked the double-positive neurons with ESR1 and cFOS (Figure. 9C). I observed that the percentage of double-positive neurons was lower in *Esr1*$^{\Delta 9/+}$ females (ESR1 + cFOS positive cells in total ESR1 cells; *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.042, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.0015) . As lower ESR1 positive neurons were present in these females, the excitation also lower compared to control group (ESR1 + cFOS positive cells in total cFOS cells; *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.013, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.033). Overall, these findings suggest that the inability to perform good maternal gestures by *Esr1*$^{\Delta 9/+}$ females might be because of lower numbers of ESR1 positive neurons in MPOA.

Normal estrus cycle among the females with reduced Estradiol levels in Esr1$^{\Delta 9/+}$ ***females***

Hypothalamic MPOA is considered a hub for several fast-acting neurotransmitters and neuropeptides, and also it has been shown that estrus state-dependent gene or protein expression could modulate the functional properties of subset of neurons (*44*). Estrus and diestrus states of females reflect changes in circulating estrogen with corresponding levels of performing sexual behaviors. Additionally, ESR1 expression in MPOA was reported be estrus dependent. Though I did not check the estrus stage of females while performing maternal behavior, the reduced ESR1 expression in MPOA made me to question whether *Esr1*$^{\Delta 9/+}$ females are maintaining a healthy estrus stage or not.

Females were monitored for at least for 3 estrus cycles continuously before estimating estradiol levels. In general, the length of estrus cycle in mice is 4-5 day long.

Proestrus and estrus stage females are considered reproductively active. I characterized the estrus stage of female mice by acquiring vaginal smears and examining their epithelial cytology under a microscope after staining with Giemsa stain (Figure 10A). All the female mice irrespective of the genotype maintained a normal estrus cycle length of 4-5 days (Figure. 10A; P= 0.442). These results suggest that 9bp-deletion does not have any effect on the reproductive status of the females.

When the third estrus cycle examination completed in all the females, at the starting of 4th cycle when the female was in proestrus stage, blood plasma was collected by cardiac puncture to estimate serum estradiol levels (Figure 10B). Estradiol levels were measured by using ELISA and I found that there was individual variability even though the sampling was done in a similar estrus stage. On average, *Esr1*$^{\Delta 9/+}$ females showed reduced Estradiol levels compared to the control group (*Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.024, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.034).

Higher Esr1 mRNA and lower protein levels in Esr1$^{\Delta 9/+}$ females

During proestrus stage, I also collected the hypothalamus to check the *Esr1* mRNA and protein levels. To determine whether the 9bp-deletion affects the *Esr1* mRNA expression, total RNA was isolated from the hypothalamus and ovary. qRT-PCR analysis showed a significantly higher expression of *Esr1* mRNA in the hypothalamus of *Esr1*$^{\Delta 9/+}$ mice (*Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.005, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.002). In contrast, the *Esr2* expression in the hypothalamus of *Esr1*$^{\Delta 9/+}$ mice were lowered when compared to that of *Esr1*$^{+/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$ mice (Figure 10C; *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.005, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.002). In the ovary, *Esr1* expression remains similar among three genotypes (p=0.521), in addition, *Esr2* expression was significantly higher in *Esr1*$^{\Delta 9/\Delta 9}$ mice (Figure 10D; *Esr1* $^{+/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.06). Then I proceeded to check ESR1 protein levels by immunoblotting. Whole protein was extracted and used for immunoblotting (Figure 11A). Whole protein

levels in each lane were quantified using gel quantification method and plotted against GAPDH. The relative ESR1 protein levels (the classic full length 65kDa) showed that *Esr1*$^{\Delta 9/+}$ females have lower expression when compared to *Esr1*$^{+/+}$ mice (Figure 11D; *Esr1* $^{+/+}$ vs *Esr1*$^{\Delta 9/+}$; P=0.002, *Esr1*$^{\Delta 9/+}$ vs *Esr*1$^{\Delta 9/\Delta 9}$; P= 0.001). These results confirm the lower ESR1 protein level in *Esr1*$^{\Delta 9/+}$ females. Interestingly, another isoform ESR1 which appears at 46kDa did not show any difference in the expression levels (P=0.461).

Overall, these results indicate that, the abnormal parental behavior in the heterozygous females is mainly due to lower ESR1 protein levels. On the contrary *Esr1* mRNA expression levels are higher in these females, this can be due to negative feedback mechanism where cell is compensating for lower protein levels. These results suggest two possibilities; either ESR1 protein translation has been affected by heterozygous combination of the alleles or protein is degraded in a faster manner after translation. Addressing the stability of the protein will help understanding of the mechanism behind lower protein levels.

DISCUSSION

ESR1 gene function is very well studied in humans. Several studies reported a long list of *ESR1* gene sequence variants -including mutations and polymorphisms correlated with

various behavioral disorders in humans. *ESR1* is prone to alternative splicing, and it was believed that splicing is the major contributor to polymorphisms in this gene. Most of the reported polymorphisms are located in noncoding regions of the genome and do not alter the amino acid sequence, hence the molecular mechanisms in changing the behavior were not well established. In this study, I screened the entire coding exons for polymorphism identification. Though there are more than 20 SNPs present among the various mouse strains, only 3 polymorphisms resulted in change in the amino acid sequence. Here, I focussed on polymorphism rs245996394 ("in-frame" deletion) in B6 mice, which is located in the GCC repeat (coding region) on the N-terminal domain of ESR1 protein and also there was a difference in GCC repeat length between wild and laboratory mouse strains.

GCC repeat length polymorphisms and role in neurological diseases

The trinucleotide repeat- GCC codes for amino acid alanine and these alanine repeats or polyalanines are predominantly found in transcription factor genes (*45*). Seminal work by Lavoie and co-workers revealed that, among all the alanine repeats present in human genome, about 30% of repeats are longer than seven alanine's in a row (i.e; AAAAAAA) and prone to polymorphisms in length (*46*). The expansion of these repeats causes neuronal dysfunction (mainly from mid-life) which leads to severe neurodegeneration (*47*). Additionally, the length of the repeat determines the magnitude of neurological and developmental disorders (*48, 49*). Alanine repeats tend to form unusual structures (in β-sheet) leading to protein mis-folding and aggregation, which explains their role in many neurodegenerative disorders. These repeats preferentially located in the N-terminal end of proteins believed to be essential for protein–protein interactions and protein conformation. The polymorphic region in the present study, contains 9 alanine repeat in the N-terminal domain of ESR1 protein in B6 which is varied in wild-derived mouse strains with 6-8 and humans with only 5 alanine

repeats. Even though the presence of alanine repeats in ESR1 was reported earlier (*46*), their importance was not established yet. Hence, in this study I focused on 9 bp polymorphism which is present in GCC or alanine repeat region of in *Esr1* gene.

Deletion of GCC repeat (9bp) in B6 resulted in significant reduction of protein levels

In my preliminary analysis, I checked whether 9bp deletion has affected the ESR1 protein structure. I predicted the dimeric form of ESR1 of three genotypes using Alphafold2 algorithm. The predicted structures showed that dimer form of $Esr1^{\Delta 9/\Delta 9}$ protein is more similar to $Esr1^{+/+}$ compared to $Esr1^{\Delta 9/+}$ and the deletion altered the amino acid composition in β-sheet formation (data not shown). As structural prediction data is completely based on sequence modelling and I could not confirm those predictions with any *in vitro* assays, this data could not provide the actual confirmation about change in protein structure. However, it provided the basic idea that heterozygous deletion of 9bp (3 alanine's) altered the protein conformation and interactions. Since structural data was not strong enough to address the changes in ESR1, I checked the protein expression levels through western blotting. A prominent reduction of hypothalamic ESR1 levels was observed in $Esr1^{\Delta 9/+}$ females where $Esr1^{\Delta 9/\Delta 9}$ maintained similar protein levels when compared to $Esr1^{+/+}$. The significant reduction in ESR1 levels was only observed at 65kD monomer length (the canonical protein). The other protein transcript which expresses at 46kD (ESR1 protein isoform without AF1 domain containing polyalanine repeat) in ESR1 protein did not show any difference in protein expression among the three genotypes. These results indicated that deletion did not affect all the ESR1 isoforms.

The lower protein levels can also be possibly addressed with ESR1 protein instability. As mentioned earlier GCC codes amino acid 'alanine. The polyalanine repeats may lead to protein misfolding and subsequent loss of function (*50*). Although the loss of function or

severity of malformation depends on length of the repeat, for example, in HOXD13 protein, both heterozygotes and homozygotes can result in severe phenotype with change in the polyalanine length (*51-53*). However, so far, I don't have any information about how the difference of polyalanine repeats in ESR1 protein affects their phenotype. If polyalanine repeats have a role in ESR1 gene, the question here is why the homozygous deletion of 9bp does not show any effect on the females like heterozygous deletion. Compared with other transcription factor genes, where polyalanine expansions resulted in lower protein levels (*41, 54*), ESR1 may function differently because of its dimeric nature. Hence, there is a possibility that similar polyalanine length in homozygotic alleles might be favourable condition than different allelic length in heterozygotes. At present based on the given results it is very difficult to address this phenomenon.

Higher Esr1 mRNA expression

The earlier finding of reduced ESR1 levels in hypothalamus led me to check whether 9bp deletion affected its receptor expression. I observed the higher *Esr1* mRNA levels upon heterozygous deletion. Earlier reports showed that, *ESR1* polymorphism rs9340799 determines the degree of cancer susceptibility by influencing the gene expression *via* transcriptional regulation (*55, 56*). In addition, another report showed that polymorphisms might alter the transcription levels of the *Esr1* (*57*), but the underlying mechanism remained unclear. There is a possibility that 9bp deletion, affected the transcriptional regulation in heterozygotes which might have resulted in higher mRNA levels.

The higher *Esr1* and lower *Esr2* mRNA expression in hypothalamus shows that both the receptors balance the estrogen functions by compensating for each other. However, there was no change in *Esr1* mRNA expression in ovary between three groups and also $Esr1^{\Delta 9/+}$ females maintained healthy estrus cycle despite lower Estradiol levels in proestrus. These

results indicate that the effect of 9bp deletion might be tissue specific or some other signalling pathway is involved. As ESR1 functions through different signalling pathway and so far, the present study can only speculate that polymorphisms in ESR1 might affect its signalling pathway which in turn decides the fate of events in brain.

Heterozygous deletion of 9bp in B6 genetic background affects pup retrieval behavior

Hypothalamic MPOA is considered as main driving region for maternal behavior. When MPOA ESR1 positive cells were optogenetically inactivated, females were unable to retrieve the pups (*14*). These observations showed that heterozygous deletion of 9 bp led to abnormal parental behavior in virgin females. In the elaborated maternal behaviors, it was observed that initially all females hesitated to approach the pups, though in later sessions they were quick in approaching and sniffing the pups. To approach 1st and 2nd pups' females took almost similar time irrespective of the genotype. However, in case of 3rd pup approach, *Esr1*$^{\Delta 9/+}$ females took longer time in approaching. Immunostaining results showed lower ESR1 expressing cells in MPOA in *Esr1*$^{\Delta 9/+}$ females. Lower expression can relate to delayed pup retrieval and higher offspring mortality in heterozygote females. I also checked for expression of neuronal excitation marker protein cFOS and found that this protein expression was low in *Esr1*$^{\Delta 9/+}$ females. Low expression can be explained because these females maintained less pup contact. These results indicate that heterozygous deletion of 9bp in B6 background is not favourable for females to perform the normal retrieving behavior. The reduced tendency to retrieve pups in heterozygote females can be correlated with the previous report on human where SNPs in *ESR1* gene resulted in harsh maternal parenting (*25*). In addition, human studies showed that, women with SNPs in *ESR1* gene showed various behavioral outcomes like psychiatric disorders (*58*), dementia (*59*), cognitive impairment (*60, 61*), sudden changes in mood (*62*) and depression (*20*). However, for the

same SNP, behavioral outcomes of each study differs from one another. For example, *ESR1* PvuII polymorphism is associated with increased Alzheimer's risk in Caucasian populations, but not in Asian populations (*63*). These variations are particularly relevant in populations consisting of different ethnicities. Overall, all these findings implicated that *Esr1* gene polymorphisms alter mRNA and protein expression levels in brain, which might have altered the estrogen signalling pathways resulting in behavioral disorders. Though present study only focussed on maternal behavior, it might be an indication that studying the polymorphisms in mice will open the more ways to understand the molecular mechanisms for change in behaviors in humans.

In conclusion, my study identified that mice from different genetic background, i.e., laboratory strain and wild derived strains differ in *Esr1* gene structure. I found a repeat number polymorphism of GCC in ESR1 coding region between B6 and MSM. This polymorphism i.e., deletion of 9bp in B6- *Esr1* gene resulted in abnormal maternal behavior. Briefly, our findings include; (i) increased pup mortality in $Esr1^{+/\Delta 9}$ (heterozygous) females, $Esr1^{+/\Delta 9}$ females showed reluctance in pup retrieval (took more time), (iii) reduced expression of ESR1 and cFOS (immediate early gene) in the MPOA of $Esr1^{+/\Delta 9}$ mice after maternal behavior implicating the reluctance in pup retrieval, (iv) higher *Esr1* mRNA expression and lower protein levels in hypothalamus of $Esr1^{+/\Delta 9}$ females, indicating the effect of polymorphism on regulation at either transcription or translation level. Altogether, this study provides a glance of how a *Esr1* genetic polymorphism has affected maternal behavior

Table 1: List of mice strains used for *Esr1* gene sequence analysis

S.No	Mice strain	Type	*Esr1* gene Coordinates	Gene Model ID
1	C57BL/6J	Inbred	Chr10:4561593-4955633 (+)	MGI_C57BL6J_1352467
2	129S1/SvImJ	Inbred	Chr10:1642184-2065710 (+)	MGP_129S1SvImJ_G0016981
3	A/J	Inbred	Chr10:1544205-1933222 (+)	MGP_AJ_G0016959
4	AKR/J	Inbred	Chr10:1574236-1937241 (+	MGP_AKRJ_G0016924
5	BALB/cJ	Inbred	Chr10:1507424-1901789 (+)	MGP_BALBcJ_G0016917
6	C3H/HeJ	Inbred	Chr10:1630001-2034933 (+)	MGP_C3HHeJ_G0016742
7	C57BL/6NJ	Inbred	Chr10:1664905-2067624 (+)	MGP_C57BL6NJ_G0017380
8	CBA/J	Inbred	Chr10:1731821-2167974 (+)	MGP_CBAJ_G0016714
9	DBA/2J	Inbred	Chr10:1540708-1923563 (+)	MGP_DBA2J_G0016822
10	FVB/NJ	Inbred	Chr10:1504071-1878661 (+)	MGP_FVBNJ_G0016817
11	LP/J	Inbred	Chr10:1605100-1986935 (+)	MGP_LPJ_G0016897
12	NOD/ShiLtJ	Inbred	Chr10:1632405-2069771 (+)	MGP_NODShiLtJ_G0016848
13	WSB/EiJ	Inbred	Chr10:1629088-2023133 (+)	MGP_WSBEiJ_G0016385
14	NZO/HlLtJ	Inbred	Chr10:1563681-1960062 (+)	MGP_NZOHlLtJ_G0017417
15	PWK/PhJ	Wild-derived	Chr10:1548497-1936520 (+)	MGP_PWKPhJ_G0016110
16	SPRET/EiJ	Wild-derived	Chr10:1743983-2169441 (+)	MGP_SPRETEiJ_G0015891
17	CAROLI/EiJ	Wild-derived	Chr10:1360444-1727110 (+)	MGP_CAROLIEiJ_G0015086
18	CAST/EiJ	Wild-derived	Chr10:1686626-2096011 (+)	MGP_CASTEiJ_G0016324

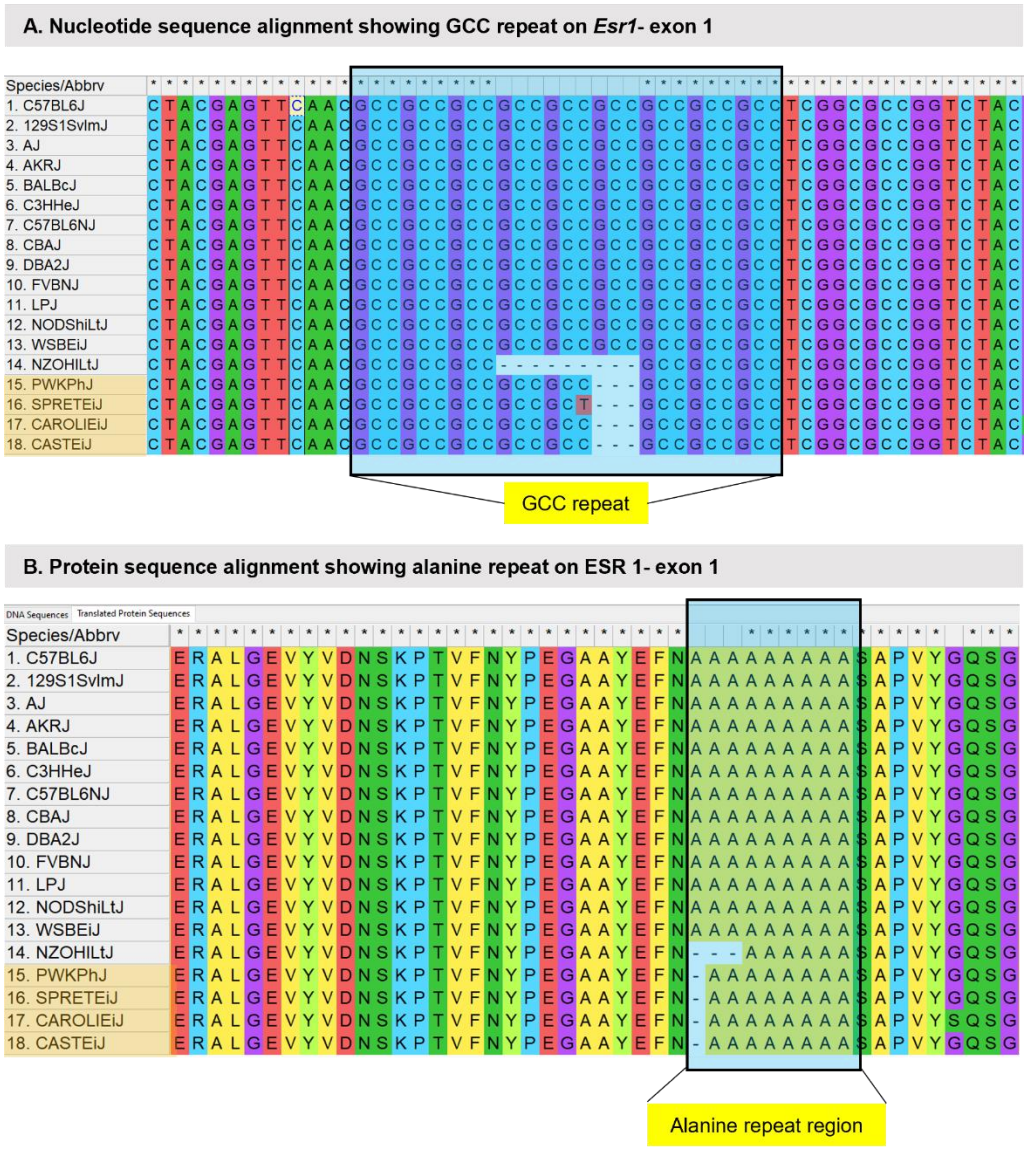

Figure 1: Selection of *Esr1* gene sequence region for polymorphism identification

A. The *Esr1* gene sequence of all 18 mice strains was obtained from MGI (mouse genome informatics; http://www.informatics.jax.org/). Nucleotide sequence alignment of *Esr1*-exon 1 showing the GCC repeat area. This GCC repeat length is shortened by one repeat (3bp) in wild derived stains (list:15-18, orange color) when compared with laboratory mice. The inbred strain, NZO/HILtJ (list:14) has the repeat shortened by 9bp. The detailed location and *Esr1* gene ID of each mice strain was listed in table 1.

B. Protein sequence alignment showing the alanine repeat region (polyalanine expansion or domains) of ESR1 protein. This polyalanine length was shortened by one alanine (A) in wild derived stains (list:15-18, orange color) when compared with laboratory mice. The inbred strain, NZO/HILtJ (list:14) has the repeat shortened by 3 amino acids.

*Sequence alignment was done in MEGA11 using MUSCLE algorithm.

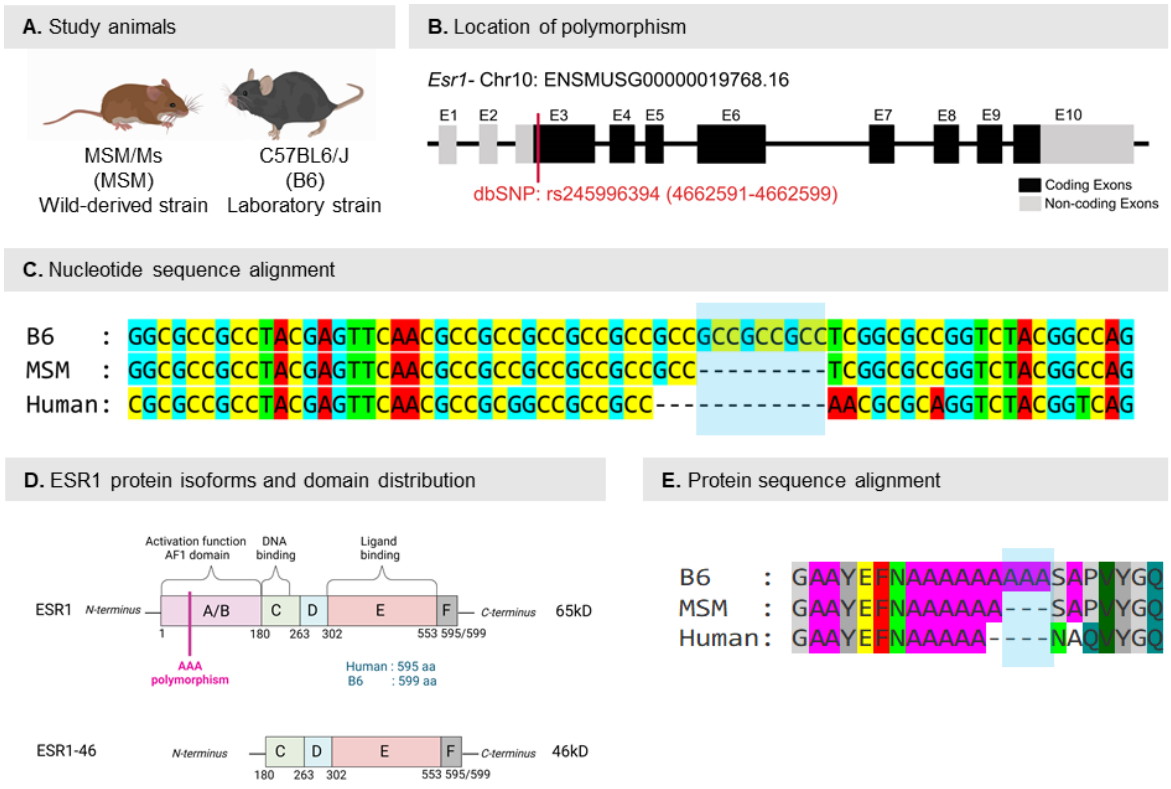

Figure 2: Location of 9bp polymorphism in *Esr1* gene and protein

A. Mice strains used in the study for polymorphism identification. MSM is wild derived and B6 is commonly used laboratory mice.

B. Location of polymorphism on the *Esr1* gene showing coding and non-coding exons. 9bp polymorphism is present in the coding exon 1. In ensemble database the 9bp polymorphism was reported as in-frame deletion rs245996394 in GCC repeat region (http://asia.ensembl.org/Mus_musculus/Transcript/Exons?db=core;g=ENSMUSG00000019768;r=10:4561593-4955614;t=ENSMUST00000105590)

C. Nucleotide sequence alignment showing the 9bp polymorphism in GCC repeat region of B6, MSM and Human.

D. Schematic view of ESR1 protein domains and location of 3 alanine's in AF1 domain. ESR1 full protein appear at 65kD molecular weight in western blot. ESR1-46 is isoform without AF1 domain which appear at 46kD molecular weight.

E. Protein sequence alignment of B6, MSM with human ESR1 protein showing the partial alignment in polyalanine domain.

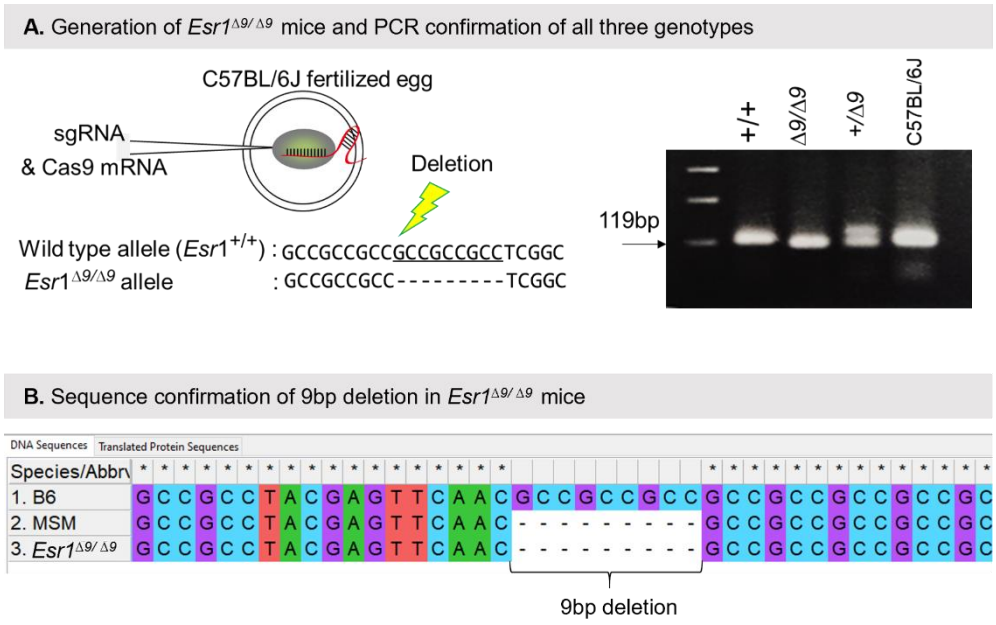

Figure 3: Generation of *Esr1*$^{\Delta 9/\Delta 9}$ mice and sequence confirmation

A. Representation of CRISPR/Cas9 genome editing. *Esr1*$^{\Delta 9/\Delta 9}$ mice were generated in B6 background by microinjecting sgRNA and Cas9 mRNA in B6 fertilized egg. PCR genotyping of wild type (*Esr1*$^{+/+}$), heterozygotes (*Esr1*$^{\Delta 9/+}$) and homozygote (*Esr1*$^{\Delta 9/\Delta 9}$) mice obtained by breeding of founder heterozygotes.

B. Sequence alignment showing the deletion of 9bp in *Esr1*$^{\Delta 9/\Delta 9}$ mice similar to MSM

* Sequence alignment was done in MEGA11 using MUSCLE algorithm

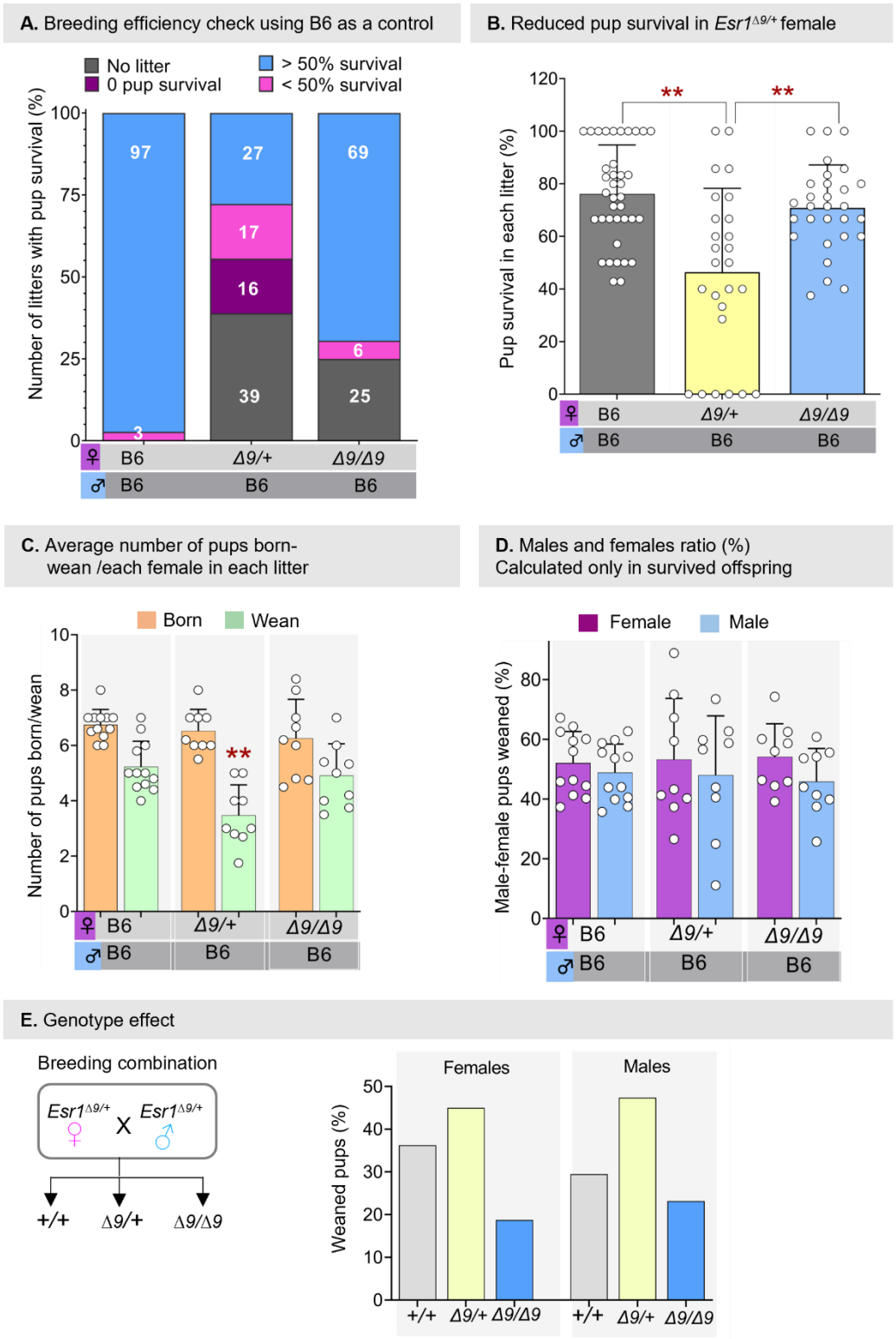

Figure 4: Genotype of pups

A. Breeding efficiency check by using different breeding combinations with B6, indicating the pup survival in each genotype. Number inside the bar indicates the percentage of breeding females falls under the respective category. On average 2-4 litter per female and total of 30-40 litter per group are considered for plotting the data. >50% means pups weaned are more than 50% and <50% means pups weaned are less than 50%.

B. Bar graph showing the statistical analysis of pup survival among the females of three genotypes. p = 0.0025 (B6 versus *Esr1* $^{\Delta 9/+)}$ and p = 0.029 (B6 versus *Esr1* $^{\Delta 9/\Delta 9)}$).

C. Average number of pups born and wean per each female. Each dot represents the average number of pups born in a single female. There was no difference in number of born pups, but in case of *Esr1*$^{\Delta 9/+}$ females' number of weaned pups decreased significantly (p = 0.0014 (B6 versus *Esr1* $^{\Delta 9/+)}$.

D. Percentage of male and female pups at the time of weaning (in each female). 80-100 pups were considered to calculate the percentage per each group.

E. Genotype effect on survived pups. Weaned females and males are plotted according to their genotype. Number of *Esr1* $^{\Delta 9/+}$ females and males used for breeding (n=6 each); total 23 litters from all mating pairs (litter number: 5, 3, 3, 5, 2, 5). Total number of pups born=171; wean=76; females;42 and males=34). Percentage was calculated for total number of female or male weaned with each genotype. Chi-square test was performed to check whether distribution of three genotypes is equal or not. There was no significance in distribution of genotypes.

*All the horizontal axis of graph showing the breeding combination of female and male. One-way ANOVA with posthoc Tukey HSD. *P<0.05

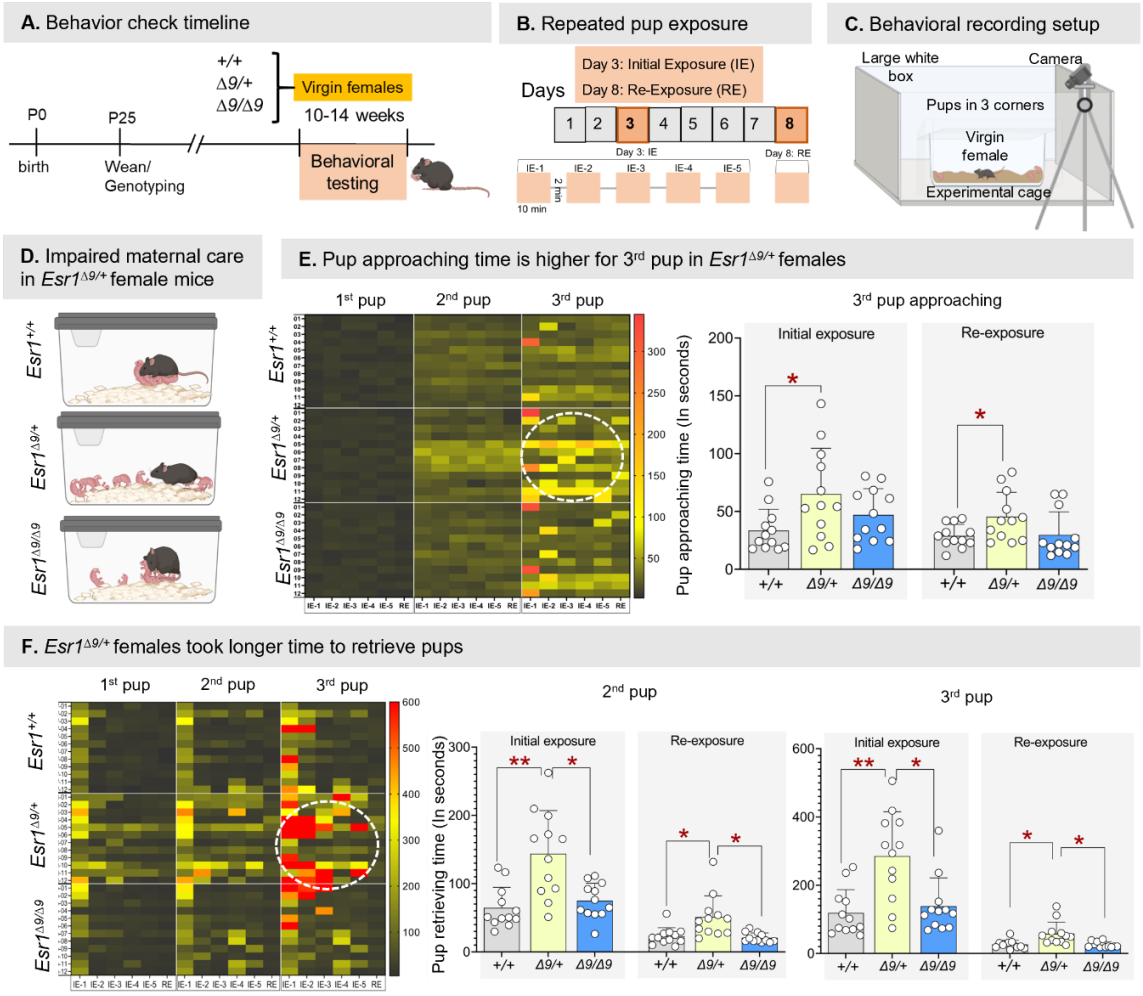

Figure 5: *Esr1*$^{\Delta 9/+}$ *showed abnormal maternal behavior*

A-C. Experimental design and behavioral recording set up. **A.** Virgin females of three genotypes (aged 10-14 weeks) were tested for pup exposure. **B.** Behavioral paradigm was designed in such a way where female will be exposed to pups repeatedly. After 2 days of habituation to the experimental cage, female will be exposed to pups on day 3 repeatedly for 5 times (10 min each with 2 min break). In each session new pups were given to female. On day 8 female will be re-exposed to new set of pups, referred as re-exposure session. **C.** Behavioral recording set up

D. Illustration showing the impaired maternal care in *Esr1*$^{\Delta 9/+}$ females. Representation of home-cage situation.

E. Heat map showing the latency to approach each pup separately in each session. Colour panel indicates (black->50 sec; yellow-> 200 sec; red-<200sec) the time taken by female to

approach the pup. Bar graph showing the 3rd pup approaching time, which is significantly higher in *Esr1*$^{\Delta 9/+}$ females than the other genotypes.

F. Latency to retrieve each pup separately in each session (heat map) and bar graph showing the 2nd and 3rd pup retrieval which is higher in *Esr1*$^{\Delta 9/+}$ females.

*Error bars ± SD; One-way ANOVA with posthoc Tukey HSD test.

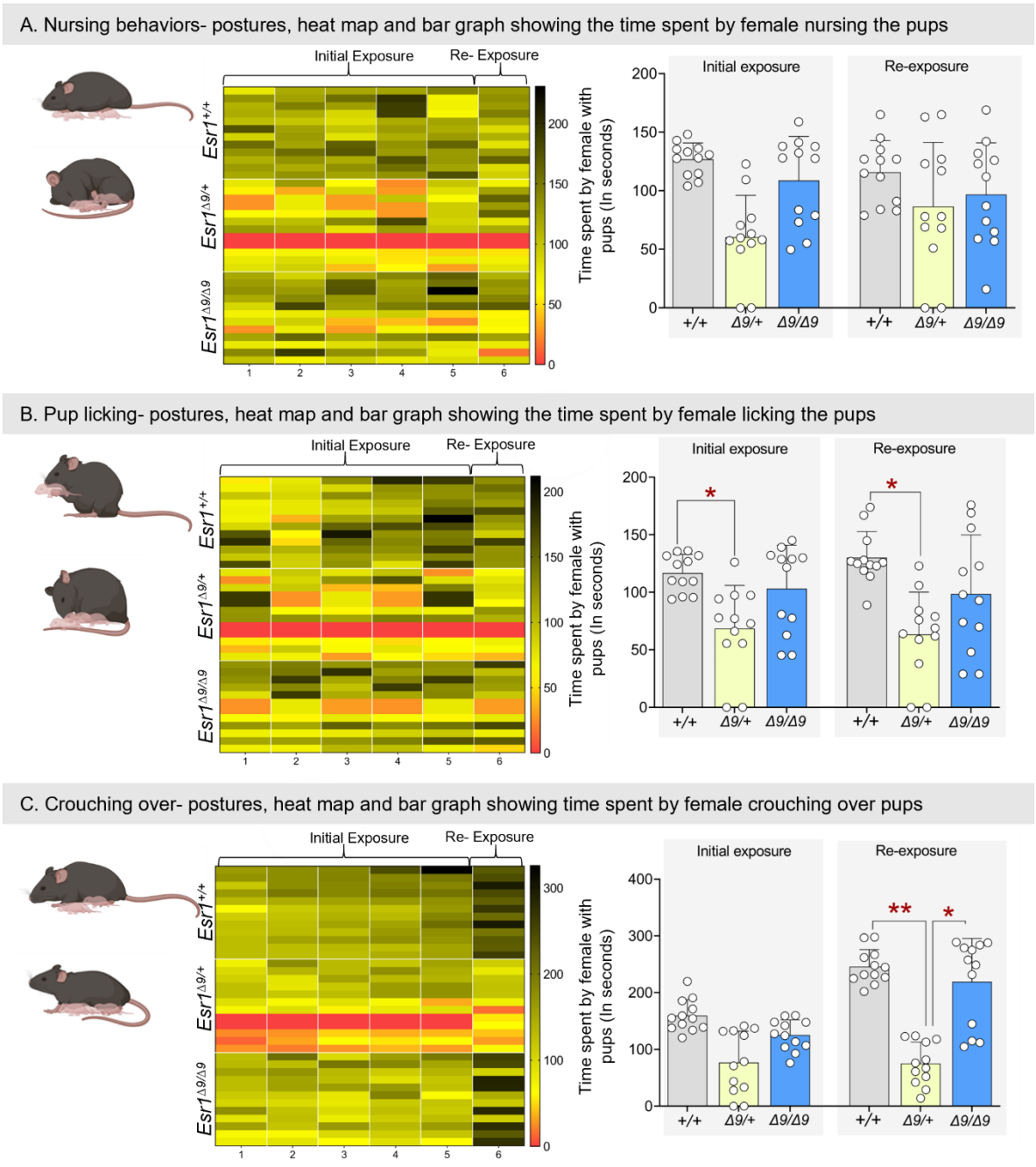

Figure 6: ***Elaborated maternal behaviors showed by females-*** The postures exhibited females while interacting with the pups determine the specific type of maternal gestures.

A. Nursing behaviors: The type of postures considered for determining the nursing behavior. The heat map showing the total time spent by the female in each session performing nursing behaviors. The bar graph showing the combined result of initial and re-exposure session which shows no statistical difference between the genotypes.

B. Pup licking behavior: Female holding the pup and continuously licking is called as pup licking behavior. The total time spent by female continuously licking the pup (at least 1 pup) was calculated and showed in the heat map for each session differently. The combined result was plotted in the bar graph which shows that *Esr1*$^{\Delta 9/+}$ females spent less time in licking the pups.

C. Crouching over the pups: Female perform hovering over the pups to provide the warmth to the pups. This kind of position considered as caring behavior however no active nursing is involved. The postures showing the hovering over the pups are calculated and plotted in a heat map showing each individual session. Bar graph showing the combined data of initial and re-exposure session. The initial exposure session has no difference between the genotypes, however in re-exposure session *Esr1*$^{\Delta 9/+}$ females spent less time with the pups/pup.

*Error bars ± SD; One-way ANOVA with posthoc Tukey HSD test.

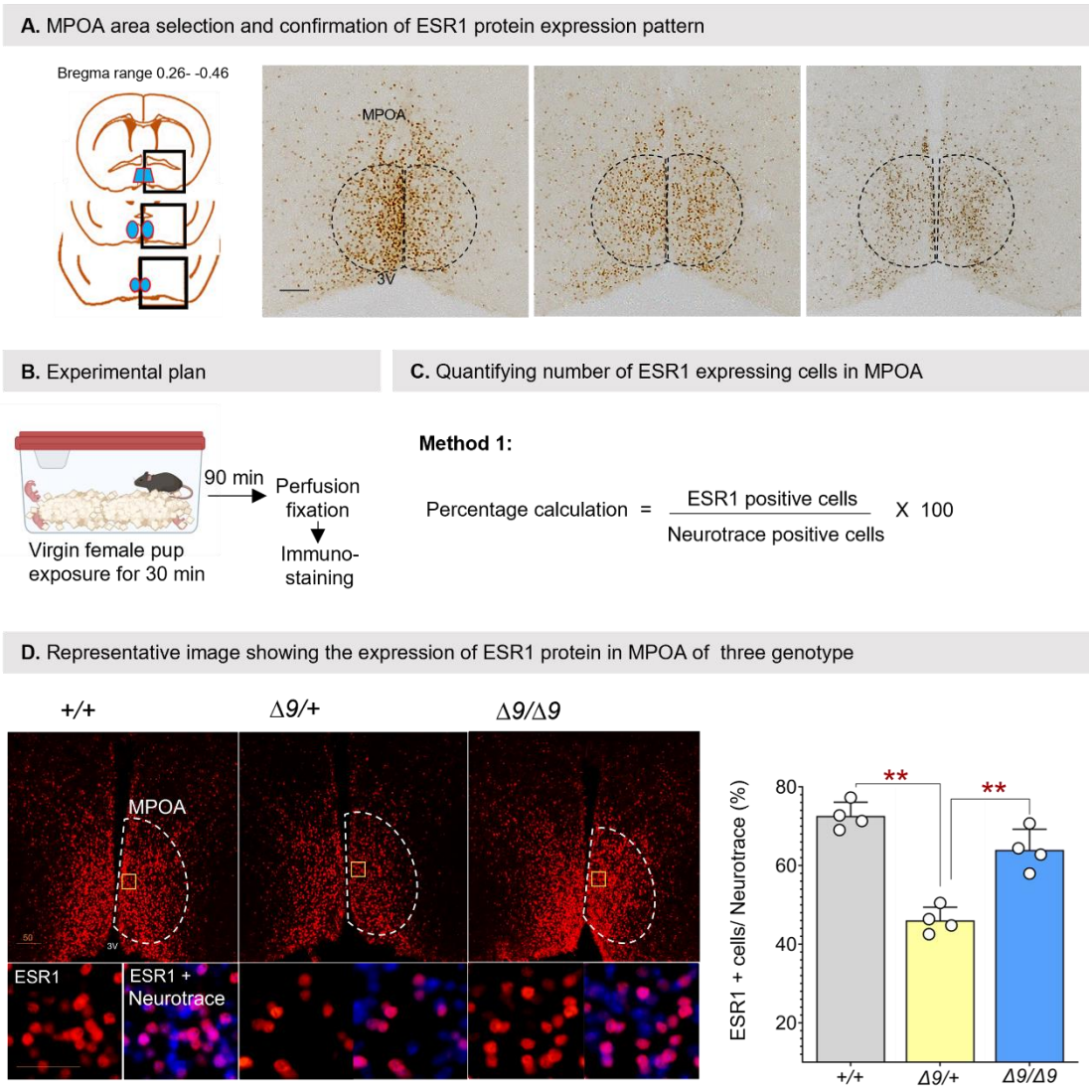

Figure 7: Expression analysis of ESR1 protein.

A. Selection of MPOA area according to brain atlas. ESR1 expression pattern confirmation through immunostaining using DAB immunostaining method.

B. Experimental plan showing the pup exposure assay. Females were given 3 pups in their home cage for 30 minutes and then cFOS analysis was performed. 30 minutes exposure was considered as optimal for detecting cFOS immunoreactive cells after pup exposure.

C. Formula showing the calculation of percentage of ESR1 expressing cells in MPIOA

D. ESR1 protein expression in three genotypes. Larger magnification image showing the quantification of NeuroTrace and ESR1 positive cells. In method 1, automatic counting of ESR1 positive cells was performed. Bar graph showing the percentage of ESR1 positive cells in whole population of NeuroTrace

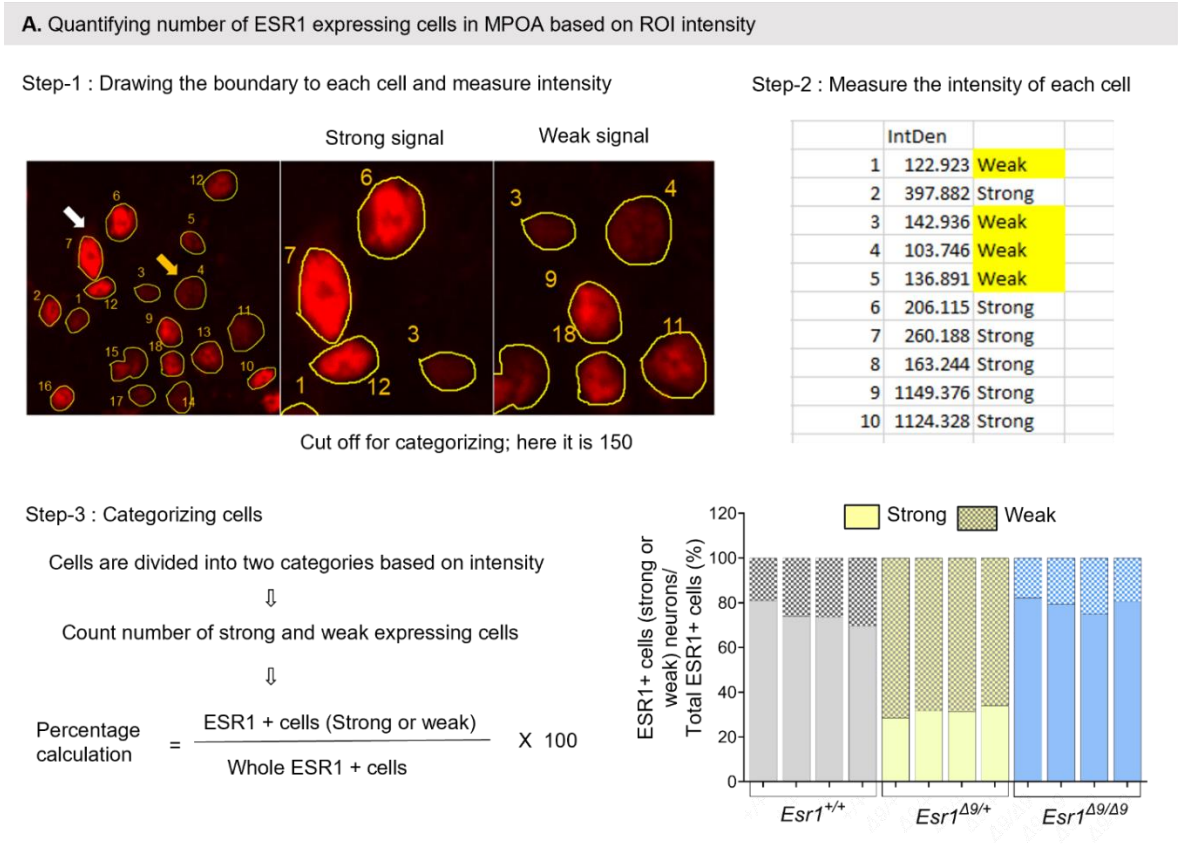

Figure 8: ROI based expression analysis of ESR1 protein.

Step-1: ROI based measurement of intensity for each cell and then cells were classified as strong and weakly expressed cells based on the intensity measurement.

Step-2: Excel showing the intensity measurement and categorizing the cells. The number of cells with strong and weak expression was noted. Marking the cell and measuring the intensity was done using the imageJ.

Step-3: Formula for calculation of percentage. At least 100 cells were taken for the analysis and percentage was calculated in the pool of whole ESR1 positive cells.

Bar graph showing the percentage of number of cells with strong and weak expression. Each bar represents each individual (n=4 for genotype). No statistical test was done to check the significant difference in the expression.

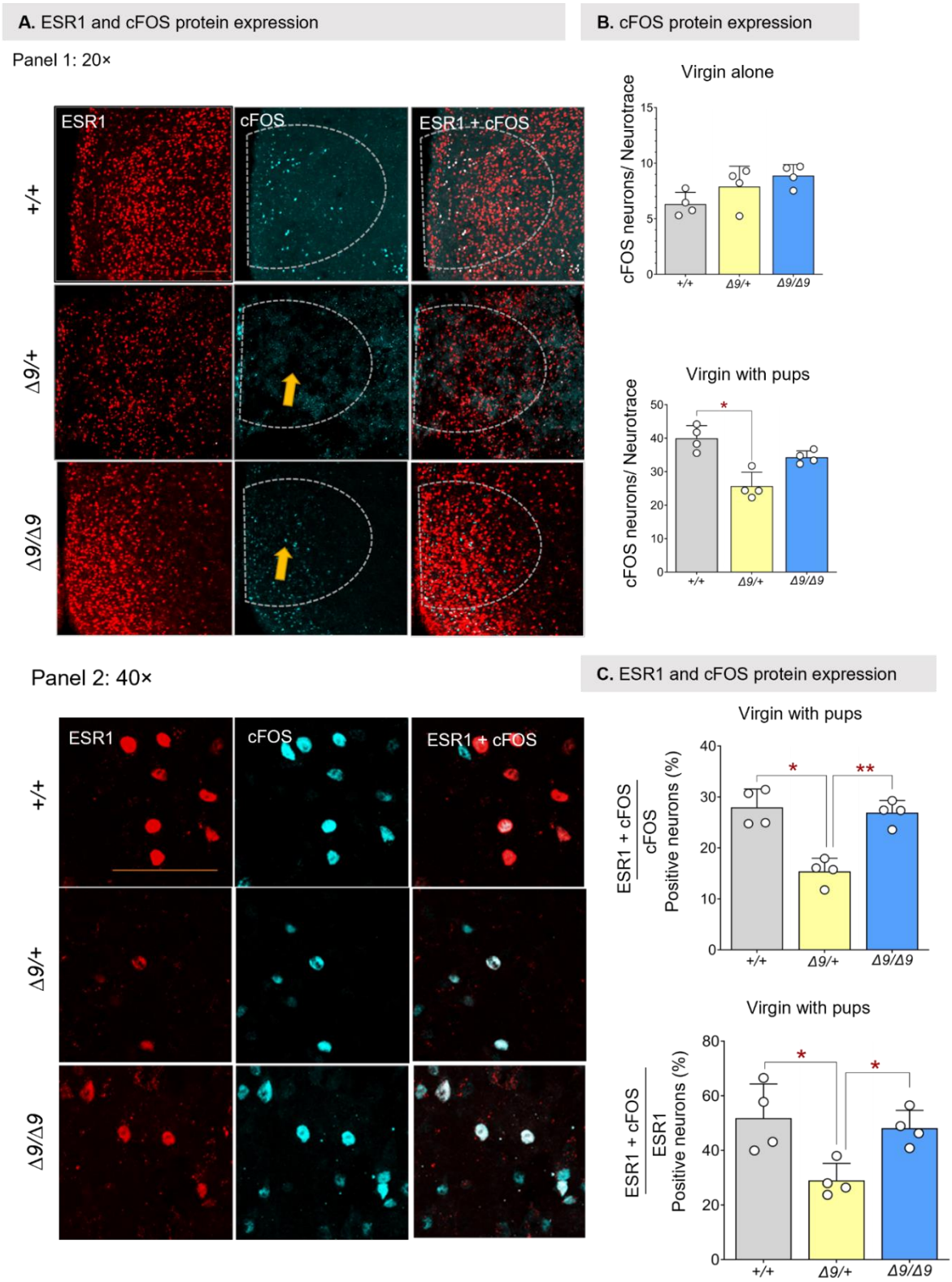

Figure 9: Expression analysis of ESR1 and cFOS proteins.

A. Representative image showing the cFOS immunostaining in three genotypes after pup exposure. Higher magnification image showing the counting of double positive cells.

B. Bar graph showing the cFOS positive cells in virgin alone and after pup exposure in NeuroTrace positive cells. When the virgin female is alone, there is no difference the number of cFOS positive neurons among three genotypes. After the pup exposure, the number of positive cFOS neurons highly increased in *Esr1*$^{+/+}$ and *Esr1*$^{\Delta 9/\Delta 9}$ females when compared to *Esr1*$^{+/\Delta 9}$ females.

C. ESR1 and cFOS double positive cells in cFOS and ESR1 positive neurons population. ESR1+cFOS/cFOS means the percentage of double positive cells of ESR1 and cFOS in whole cFOS positive cells.

ESR1+cFOS/ESR1 means the percentage of double positive cells of ESR1 and cFOS in whole ESR1 positive cells.

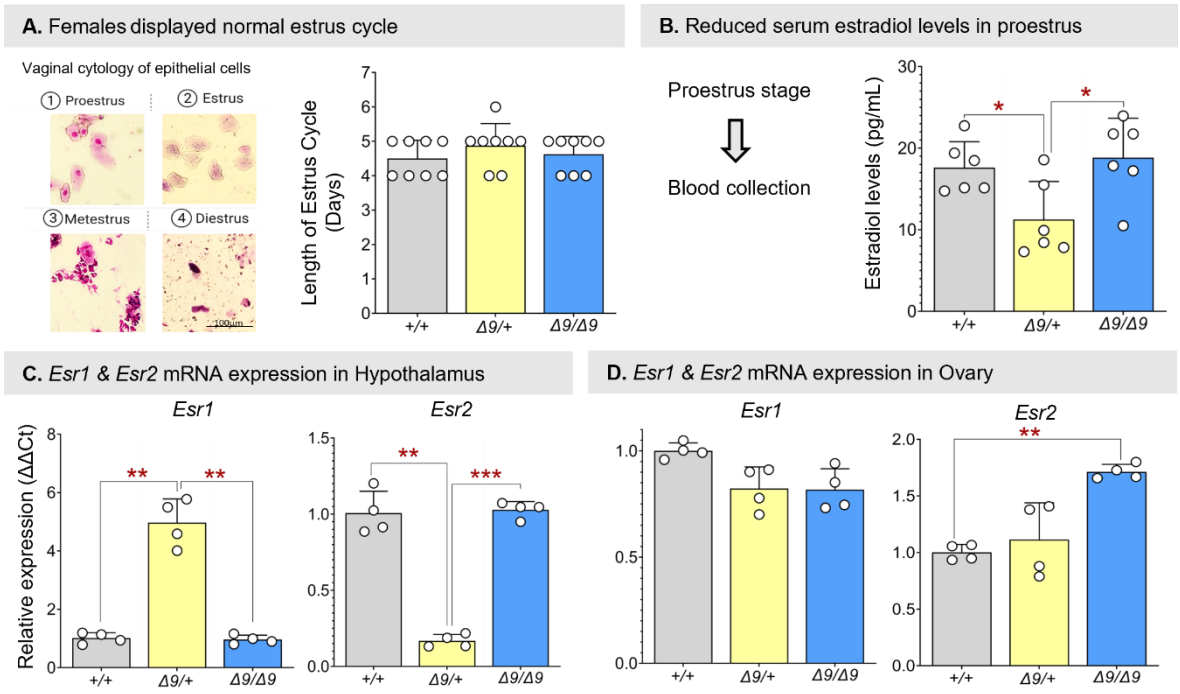

Figure 10: *Esr1*$^{\Delta 9/+}$ females display abnormal increase in *Esr1* mRNA expression

A. Estrus cycle check timeline. The mouse estrus cycle is usually 4-5 days long consisting of 4 stages as indicated. Estrus stage was monitored for n=24 females (8 for each genotype). Histogram representing the length of estrus cycle in each female. Females are considered sexually perceptive if they are in either proestrus or estrus stage.

B. Serum was isolated from females in proestrus stage. Histogram showing the serum estradiol levels checked by ELISA

C. qRT–PCR analysis of mRNA expression levels of *Esr1* and *Esr2* mRNA normalized to house-keeping gene *Gapdh* of 11–14-week-old virgin females. Each dot represents one individual female. *Esr1* mRNA expression was significantly higher in *Esr1*$^{\Delta 9/+}$ females whereas *Esr2* expression decreased significantly in hypothalamus.

D. In ovary, *Esr1* expression did not differ between the genotypes, in addition, *Esr2* expression was significantly increased in *Esr1*$^{\Delta 9/\Delta 9}$

All the data were compared with each value for statistical analysis. One-way ANOVA followed by post-hoc Tukey analysis was applied. Values were checked for normal distribution. All data are presented as mean ± SD. N=4 for each genotype.

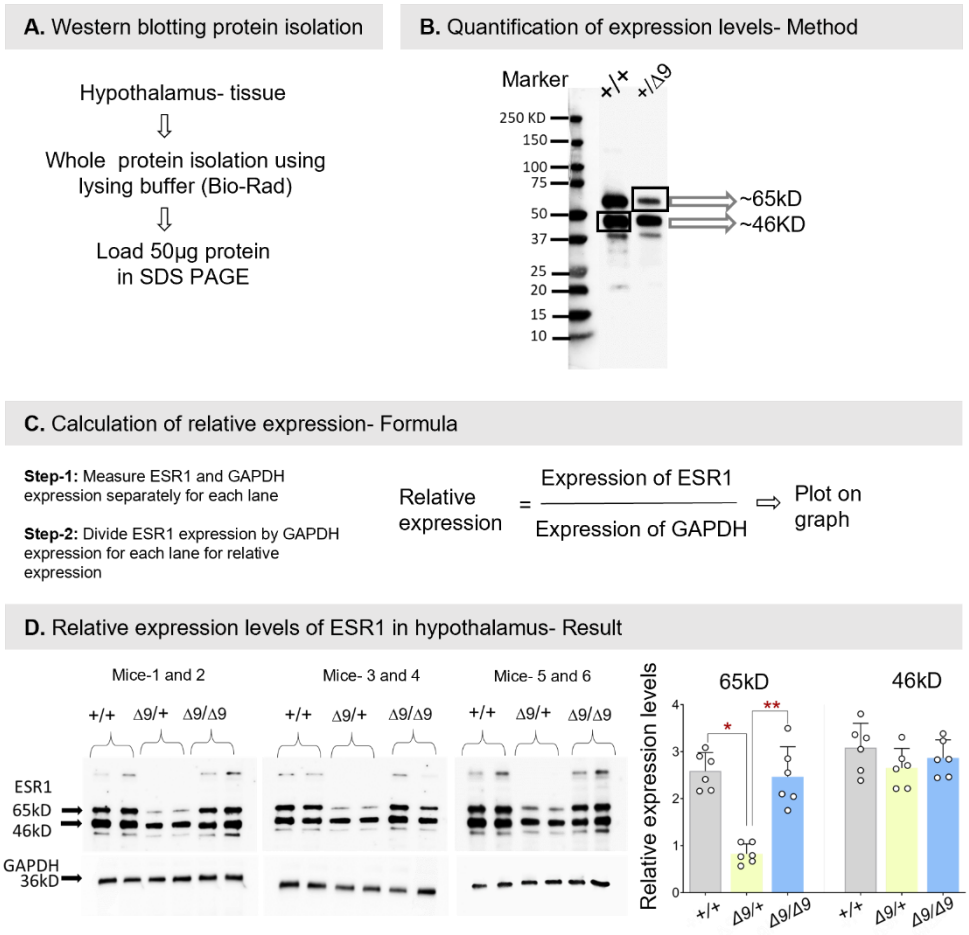

Figure 11: Quantification of ESR1 proteins levels by western blotting

A. Whole protein isolation from hypothalamus and ~50µg protein was loaded in SDS-PAGE and blotted with ESR1, following stripping reblotted with GAPDH.

B. The molecular weight of ESR1 monomer is 65kD. In hypothalamus, the ESR1 protein expression reported to be seen at different molecular weights. For analysis I measured the intensity of 65 and 46kD band using ImageJ-gel quantification tool. Black box around the band was representation for how the intensity was measured.

C. Formula used for quantification of relative expression. ESR1 expression level are normalized by using the GAPDH protein levels.

D. Western blot image showing the expression of ESR1 and GAPDH. In each set two per each genotype was shown. Overall, three sets with 6 mice were checked for expression levels. Bar graph showing the expression levels of ESR1 protein (n=6). The marker showed in the B was used for estimating the molecular weight.

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