Turner Bradley (Orcid ID: 0000-0001-6580-7655)

Exploring germline recombination in Nestin-Cre transgenic mice using floxed androgen receptor

Victoria M. McLeod[1], Brittany Cuic[1], Mathew D.F. Chiam[1], Chew L. Lau[1], Bradley J. Turner[1,2]*

[1]*Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, VIC 3052, Australia*

[2]*Perron Institute for Neurological and Translational Science, Queen Elizabeth Medical Centre, Nedlands, WA 6150, Australia*

* Corresponding author

Bradley J. Turner

Florey Institute of Neuroscience and Mental Health

University of Melbourne

30 Royal Parade

Parkville, VIC, Australia, 3052

Tel: +61 3 9035 6521, Fax: +61 3 9035 3107

Email: bradley.turner@florey.edu.au

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Abstract:

The Cre-loxP strategy for tissue selective gene deletion has become a widely employed tool in neuroscience research. The validity of these models is largely underpinned by the temporal and spatial selectivity of recombinase expression under the promoter of the Cre driver line. Ectopic Cre-recombinase expression gives rise to off-target effects which can confound results and is especially detrimental if this occurs in germline cells. The Nestin-Cre transgenic mouse is broadly used for selective gene deletion in neurons of the central and peripheral nervous systems. Here we have crossed this mouse with a floxed androgen receptor (AR) transgenic to generate double transgenic neuronal ARKO mice (AR^{flox}::NesCre) to study germline deletion in male and female transgenic breeders. In male AR^{flox}::NesCre breeders, a null AR allele was passed on to 86% of progeny regardless of the inheritance of the NesCre transgene. In female $AR^{flox/wt}$::NesCre breeders, a null AR allele was passed on to 100% of progeny where AR^{flox} was expected to be transmitted. This surprisingly high incidence of germline recombination in the nestin-Cre driver line warrants caution in devising suitable breeding strategies, consideration of accurate genotyping approaches and highlights the need for thorough characterisation of tissue-specific gene deletion in this model.

The Cre-loxP system of studying cell-type specific effects of gene activity has become an effective and widely used tool for gene deletion in the central nervous system (CNS) (Tsien et al., 1996). These conditional knockout mice are generated by breeding floxed mice (having the gene of interest flanked by LoxP sites) with Cre mice (expressing Cre-recombinase driven by a cell-specific promoter). There are over 300 Cre tool lines now available from repositories, however, one of the main drawbacks of this technology remains - the extensive and often poorly characterised off-target gene deletion occurring in both somatic and germ cells (Harno, Cottrell, & White, 2013; Schmidt-Supprian & Rajewsky, 2007). This is especially complex when recombination arises from transient expression of Cre during development which is not detectable in adult tissue. Understanding these limitations is essential to accurately interpreting tissue-specific gene functions and adopting appropriate breeding strategies to minimise the likelihood of recombination occurring in the germ cells.

Nestin is an intermediate neurofilament protein, originally reported as a marker of neuroepithelial stem cells. The nestin-Cre transgenic mouse was first described by Tronche and colleagues (Tronche et al., 1999), a mouse expressing Cre under the rat nestin promoter and enhancer, which aimed to conditionally express Cre-recombinase in neuronal and glial precursor cells. The widespread use of this model in the neuroscience field has now led to reported evidence of multiple secondary expression sites, including the kidney and somite-derived tissues (Dubois, Hofmann, Kaloulis, Bishop, & Trumpp, 2006) and pancreas (Delacour, Nepote, Trumpp, & Herrera, 2004), with an undesirable disrupted metabolic phenotype (Declercq et al., 2015). Here we have used the floxed androgen receptor (AR) mouse line to study deletion patterns when crossed with this nestin-Cre line. AR represents an ideal floxed gene candidate for tracking germ line deletion. Firstly, the *Ar* gene is located on the X-chromosome whereby one allele is inherited by each parent in females, and males are hemizygous with maternal inheritance of their X-chromosome. Secondly, global AR deletion in

genotypic males is easily identifiable as mice appear phenotypically female. We report that both male and female mice carrying the AR^{flox} and nestin-Cre transgenes transmit a very high incidence of germline recombination to progeny.

To examine recombination, we paired AR^{flox} females with nestin-Cre males, generating the expected F1 progeny (Figure 1a) with PCR products from a tail biopsy distinguishing between AR^{wt} (wildtype) and AR^{flox} alleles (Figure 2a). In Figure 1, we report the floxed allele as X^{fl} to reflect the inheritance pattern of the X chromosome carrying *Ar*. Hemizygous floxed male mice carrying the nestin-Cre transgene, AR^{flox}::NesCre, have deletion of AR protein almost entirely from the CNS as evident from brain immunoblot (Figure 3a,b). When these males are paired with $AR^{wt/wt}$ females, all male progeny inherit a maternal AR^{wt} allele as expected and females would theoretically be heterozygous for the AR^{flox} allele paternally inherited (Figure 1b). PCR genotyping of tail biopsy revealed some female progeny presenting a single AR^{wt} band (Figure 2b). We quantified the *Ar* level in these tail DNA samples using qPCR analysis and revealed a single copy of *Ar* present in these females compared to two copies in wild-type female mice. Out of possible 51 female mice, only 7 inherited the paternal AR^{flox} allele, hence, male breeders showed 86% frequency of AR deletion in the germline (analysis of 14 litters, 95 progeny; Table 1).

Heterozygous floxed female mice carrying the nestin-Cre transgene, $AR^{flox/wt}$::NesCre, have deletion of AR from 50% of CNS neuronal tissue as evident from brain immunoblot (Figure 3c,d). The breeding strategy used in Figure 1c crossed these heterozygous females with an AR^{flox} male with the intent of creating a homozygous AR floxed female carrying the nestin-Cre transgene. This mating pair was anticipated to produce an equal proportion of AR^{wt} and AR^{flox} male progeny and females either homozygous or heterozygous for AR^{flox} allele, irrespective of the nestin-Cre transgene inheritance

(Figure 1c). PCR analysis of tail biopsy revealed mice absent for the AR allele in tail DNA, presenting with a female phenotype (Figure 2c). These mice genotyped positive for the Y chromosome and the externalised female appearance (identified by a shorter genito-anal distance and prominent mammary gland formation) similar to genetic females, was due to global deletion of AR (Figure 2d). All female progeny genotyped as having only AR^{flox} detectable (samples C17 and C18, Figure 2c) were further analysed for *Ar* copy number by qPCR analysis and mice were revealed to be hemizygous for the *Ar* allele. Out of a possible 49 male and female progeny, all 49 had a deletion of the maternally inherited AR^{flox} allele, hence, female breeders showed 100% frequency of AR deletion in the germline (analysis of 17 litters, 94 progeny; Table 1). AR deletion occurred in progeny both carrying the nestin-Cre transgene and those without, confirming that the AR gene was most likely excised in the germ cells prior to zygote formation and therefore independently of nestin-Cre inheritance.

Given the high frequency of recombination imparted on germ cells of AR^{flox}::NesCre carrying mice, we determined the impact of ectopic nestin-Cre transgene driven deletion of AR from testis and ovary. AR is clearly present in the Sertoli, Leydig and peritubular myoid cells within the testis as described (Deng, Chen, Zhang, & Wang, 2018), although evidently reduced in the AR^{flox}::NesCre mice, particularly in the Leydig cell clusters (Figure 4a). Testis showed a 35% reduction in AR protein expression (Figure 4b,c) and a 62% deletion of *Ar* DNA (Figure 4d). The discrepancy between *Ar* gene deletion and AR protein reduction is likely due to the confounding effect of increased circulating testosterone in the mice, leading to stabilisation of the AR protein (Kemppainen, Lane, Sar, & Wilson, 1992). This occurs due to deletion of AR in the hypothalamus which disinhibits the regulatory feedback control of AR production in the testis. The deletion of AR from either Sertoli, or Leydig cells results in infertility and arrested spermatogenesis (Chang et al., 2004; Xu et al., 2007). We noted reduced breeding capacity among these AR^{flox}::NesCre males with fewer litters produced and delayed production of the first litter

(Table 1). AR does not appear to be expressed in spermatocytes within testis (Figure 4a) and germ cell deletion of AR has reportedly no effect on fertility or sperm count (Tsai et al., 2006). In the ovary, AR is expressed in theca interstitial cells (Ma et al., 2017), granulosa cells (Sen & Hammes, 2010) and in oocytes (Li, Schatten, & Sun, 2009) as previously described (Figure 4e). No observed loss in *Ar* DNA or AR protein was detected in $AR^{flox/wt}$::NesCre ovary (Figure 4f-h). While AR deletion from oocytes does not impact fertility or ovarian morphology (Sen & Hammes, 2010), the presence of AR^{wt} allele in heterozygous floxed females may be responsible for the detectable AR signal observed in some oocytes. AR protein expression in adult tissues does not identify possible *Ar* deletion in tissues which don't express the AR protein, such as the male germline cells. It appears that recombinase activity driven by the Nestin promoter is responsible for the deletion of AR in germ cells within both sexes. Nestin expression occurs in stem Leydig cells within the testis, detected in a transgenic mouse model expressing GFP driven by the nestin promoter (Jiang et al., 2014). Leydig cells are the primary source of androgen production in testis and this may explain the reduced level of AR observed within the testis and also specifically within the Leydig cell population of adult AR^{flox}::NesCre mice. Interestingly in the Nes-GFP mice, nestin was not detected in the seminiferous tubules of these mice at 7 days old (Jiang et al., 2014). The Jackson Laboratory Cre Repository contains an extensive database mapping expression patterns of Cre lines during development using Cre reporter strains, such as the lacZ reporter. Recombinase activity was detected in the nestin-Cre line oocytes at P7 and seminiferous tubules, spermatocytes and spermatogonia in P56 males (https://www.jax.org/research-and-faculty/resources/cre-repository).

Germline recombination is emerging as a common occurrence among Cre lines used for studying nervous system targets. Synapsin I, another commonly used promoter to drive neuronal-specific Cre-expression, was also reported to have ectopic Cre activity in the testis, with 63% of progeny from male

SynCre::flox/flox mice showing germline recombination which did not occur in progeny from female SynCre::flox/flox mice (Rempe et al., 2006). Likewise in the parvalbumin-Cre line, Pvalb-2A-Cre, undesired germline recombination was present in 50% of progeny arising from a male Cre-flox parent and only 5% occurring from a female parent (Kobayashi & Hensch, 2013). The calcium/calmodulin-dependent protein kinase IIα (CAMKIIα)-Cre mouse showed differential germline recombination in male parents when bred with two different floxed lines, resulting in 72% and 98% of progeny exhibiting global deletion, for each floxed transgenic, respectively (Choi et al., 2014). Conversely, human glial fibrillary acidic protein (hGFAP)-Cre transgenic mice exhibited germline recombination more commonly in progeny from female Cre-flox parents (Zhang et al., 2013). Evidence of germline deletion in the nestin-Cre transgene has been reported by others (Friedel, Wurst, Wefers, & Kuhn, 2011; Harno et al., 2013; Zhang et al., 2013). However, this is the first detailed characterisation of the high frequency occurrence of germline recombination in all progeny from both male and female Cre-flox carrying parents. These studies also suggest that variation occurs across different Cre-loxP transgenic pairings which may arise from differences in accessibility of loxP sites for Cre-recombination.

This study and others highlight the importance of accurate Cre-recombinase detection methods, including sensitivity of various Cre-driven reporter lines, considerations on age of mice and stage of development of different cell populations, in collectively being able to understand the expression of Cre outside its intended cell target. These factors may all critically impact the reliability of data acquired from conditional knockout models. Undetected germline recombination may further confound the interpretation of results, especially where an easily identifiable phenotype is not apparent in constitutive knockouts or possible mosaic expression in heterozygous KO mice.

Knowledge of these major confounds may help guide downstream experimental breeding approaches and more accurate genotyping to correctly interpret experimental outcomes.

In conclusion, we have illustrated that nestin-Cre mice crossed with floxed mice produce aberrant germline recombination excising the floxed allele within male and female germline cells with remarkable efficiency. In the case of our target AR^{flox} transgene, the deletion of AR in these germ line cells was not observable in adult mice and did not produce an adverse phenotype, however, this germline deletion has important consequences for any downstream breeding strategies and alternatively, presents a viable strategy to produce constitutive ARKO mice.

Methods:

Mice

All animal experimentation was conducted in accordance with the Australian National Health and Medical Research Council published Code of Practice and approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (approval number 16-001-FINMH). Transgenic AR^{flox} mice (B6.129S1-$Ar^{tm2.1Reb}$/J line, stock number 018450, Jackson Laboratory) (Chakraborty et al., 2014) and transgenic nestin-Cre mice (B6.Cg-Tg(Nes-cre)1Kln/J line, stock number 003771, Jackson Laboratory) (Tronche et al., 1999). Mice were maintained on a C57BL/6 background under standard 12 h light/dark housing conditions and breeding consisted of trio containing one male and two females. Genotyping was conducted on tail biopsy collected after P8. Mice for tissue collection were litter-matched and group housed until P60-P100. Animals were killed by sodium pentobarbitone overdose (100 mg/kg, i.p.) and fresh tissue was collected and snap frozen in dry ice for biochemical analysis. Testis and ovary were frozen in embedding media immersed into precooled isopentane and stored at -80 °C until IHC processing.

Genotyping & PCR

Genomic DNA was extracted from tail biopsy and PCR performed using REDExtract-N-Amp™ Tissue PCR Kit (Sigma) according to the manufacturer's protocol. The following primers sequences were used in the study and obtained from Jackson Laboratory PCR protocols: *Ar*^{fl} (15320) forward 5'- AAA ATG CCT CCT TTT GAC CA -3', *Ar*^{fl} (15321) reverse 5'- AAG ATG ACA GTC CCC ACG AG -3'; *Cre* (oIMR1084) forward 5'- GCG GTC TGG CAG TAA AAA CTA TC -3', *Cre* (oIMR1085) reverse 5'- GTG AAA CAG CAT TGC

TGT CAC TT -3'; *Ymt* (9369) forward 5'- CTG GAG CTC TAC AGT GAT GA -3', *Ymt* (9370) reverse 5'- CAG TTA CCA ATC AAC ACA TCA C -3'; Internal positive control (oIMR7338) forward 5'- CTA GGC CAC AGA ATT GAA AGA TCT -3', Internal positive control (oIMR7339) reverse 5'- GTA GGT GGA AAT TCT AGC ATC ATC C -3'.

RT-qPCR

DNA from tail as previously extracted was used here with SYBR® Green Extract-N-Amp™ PCR ReadyMix™ (Sigma) and performed as detailed below. DNA from testis and ovary was extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen) according to manufacturer's protocol. Primer sequences were: Ar^{ex1} forward 5'- AAG CAG GTA GCT CTG GGA CA -3', Ar^{ex1} reverse 5'- GAG CCA GCG GAA AGT TGT AG -3'; and internal control *DAG1* forward 5'- CCA AGG AGC AGA TCA TAG GGC -3', *DAG1* reverse 5'- AGA GCA TTG GAG AAG GCA GG -3'. RT-qPCR was performed as previously described (McLeod et al., 2019). Relative expression of the *Ar* gene was determined using the $2^{-\Delta\Delta Ct}$ method. Data were analysed by Student's t-test comparing genotype using GraphPad Prism 8.0 software (San Diego, CA, USA) with $P<0.05$ taken as statistical significance. Data are presented as mean ± SEM.

Immunoblotting

Whole brain, testis and ovary lysates were prepared using previously described methods (McLeod et al., 2019). Protein lysates from brain (50 µg), testis (20 µg) and ovary (20 µg) were electrophoresed through TGX Stain-Free™ FastCast™ Acrylamide Kit handcast gels (Bio-Rad Laboratories, NSW, Australia) and transferred onto PVDF membrane at 20 V overnight at 4 °C. Total protein transferred

onto the membrane was imaged on a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, NSW, Australia) before 1 h blocking in 5% low-fat milk powder in TBST. Immunoblots were probed with rabbit primary antibody against AR (1:1000, Abcam, cat# ab133273) in SignalBoost™Immunoreaction Enhancer Kit (Merck Millipore, Cat# 407207) overnight at 4 °C. Secondary antibody, StarBright™ Blue 700 goat anti-rabbit secondary antibody (1:5000, Bio-Rad, Cat# 12004161) was incubated for 1 h at room temperature and blots were imaged on the ChemiDoc™ MP (Bio-Rad Laboratories). Protein band intensity was analysed using Image Lab 6.0 software (Bio-Rad) and AR protein normalised against total lane protein and expressed relative to the averaged AR^{flox} group. Statistical analysis was performed as stated above.

Immunohistochemistry

Testis and ovary were cryosectioned at 12 µm and mounted onto poly-L-lysine coated slides. Sections were fixed in 10% neutral buffered formalin for 10 minutes, washed, and blocked in 10% normal donkey serum (NDS) in PBS containing 0.3% Triton-X 100 for 1 h room temperature. Primary antibodies were incubated for 48h at 4 °C in PBS containing 6% NDS and 0.3% Triton-X 100; rabbit anti-AR (1:200, Abcam, cat# ab133273), rat anti-TRA98 (1:200, Abcam, cat# ab82527), goat anti-GDF-9 (15 µg/ml, Invitrogen, cat# PA5-47924) and rat anti-laminin (1:400, Abcam, Cat# ab11576). Secondary antibodies were incubated for 1 h at room temperature in the same diluent: anti-rabbit Alexa Fluor®-488 (1:200, Jackson ImmunoResearch Cat#, 711-545-152), anti-rat DyLight®-550 (1:200, Invitrogen, Cat # SA5-10027), anti-goat DyLight®-550 (1:400, Thermo Fisher Scientific Cat# SA5-10087), and anti-rat Alexa Fluor®-647 (1:200, Jackson ImmunoResearch, Cat# 712-605-153). For testis tissue dual staining with rat primary antibodies; anti-TRA98 was detected by anti-rat DyLight®-550 and followed

by a 1 h room temperature blocking step using anti-rat Fab fragment (1:30, Jackson ImmunoResearch, Cat # 712-007-003), prior to overnight incubation with the second primary antibody, anti-laminin, which was detected with anti-rat Alexa Fluor®-647secondary. Each image was comprised of a 10x 1 µm Z-stack captured on a Leica SP8 confocal microscope.

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Figure Legends

Figure 1. Nestin-Cre mice show paternal and maternal germline recombination. (a) Schematic of breeding strategies using nestin-Cre and AR^{flox} transgenics to generate male and female breeders with a floxed *Ar* in nestin- promoter driven tissue. (b) Expected and observed progeny from hemizygous male AR^{flox}::NesCre (denoted as a floxed *Ar* carrying X chromosome, X^{fl} Y) mice crossed with female wildtype $AR^{wt/wt}$ mice (denoted as normal AR carrying homozygous X chromosomes, X X). Unexpected genotypes resulting from germline recombination are indicated after the dashed line. The number of progeny per genotype, irrespective of Cre status, out of total progeny are indicated in parentheses. Percentage germline recombination is delineated from the unexpected observed to expected observed genotypes. (c) Expected and observed progeny from heterozygous female $AR^{flox/wt}$::NesCre (denoted as a floxed *Ar* carrying heterozygous X chromosome, X X^{fl}) mice crossed with male AR^{flox} mice. Unexpected genotypes resulting from germline recombination are indicated after the dashed line and percentage germline recombination is delineated from the unexpected observed to expected observed genotypes.

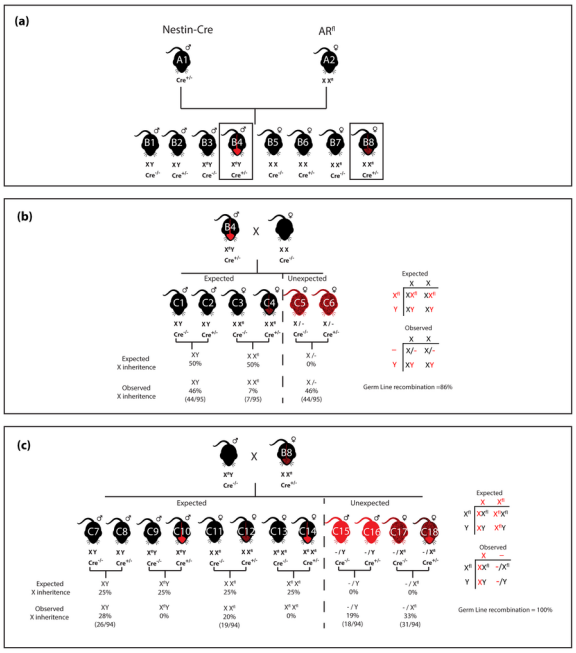
Figure 2. PCR genotyping products observed from the nestin-Cre crossed with ARflox breeding strategies in figure 1. (a) AR^{flox} male crossed with nestin-Cre female and resultant F1 progeny. Wildtype *Ar* allele presents as 164 bp, distinguishable from floxed *Ar* allele at ~230 bp. Cre transgene is detected as a ~100 bp product. (b) F1 breeder, male AR^{flox}::NesCre, and observed F2 progeny presenting from figure 1b. (c) F1 breeder, female $AR^{flox/wt}$::NesCre, and observed F2 progeny presenting from figure 1c. Males C15 and C16 had no *Ar* product in the presence of positive internal control products and were confirmed to have a Y chromosome present; Ymt product. (d) The

appearance of genetic male and female mice. Male mice absent for *Ar* showed female external genitalia, as observed by the shortened genito-anal distance compared to phenotypic males.

Figure 3. Nestin-Cre driven deletion of *Ar* in brain tissue reflects abolishment of AR protein. (a) Immunoblot of brain AR protein in male AR^{flox}::NesCre shows complete abolishment, (b) quantification of protein level against Cre-negative AR^{flox} male mice. (c) Immunoblot of brain AR protein in female heterozygous AR deleted, $AR^{flox/wt}$::NesCre, mice, (d) quantification of protein level against Cre-negative $AR^{flox/wt}$ mice shows approximately 50% reduction in AR protein as expected. Data represents mean ± SEM, n=4-5 mice per group. ***P*<0.01, *****P*<0.0001 significantly different to Cre-negative counterpart by Student's t-test.

Figure 4. AR protein is reduced in the testis of nestin-Cre driven *Ar* deleted males. (a) AR nuclear staining present in the testis of Cre-negative AR^{flox} and AR^{flox}::NesCre mice. Strong presence of AR is detectable in the Sertoli cells (s) located inside the basement membrane of the seminiferous tubule, the Leydig cells (l) located in the interstitium and the peritubular myoid cells (m) surrounding the seminiferous tubules. The testicular germ cells identified by TRA98 marker are devoid of AR protein. AR staining is noticeably decreased in AR^{flox}::NesCre testis, particularly a reduced presence of AR positive Leydig cells. (b) Immunoblot and (c) quantification of testis AR protein in AR^{flox}::NesCre mice compared to Cre-negative AR^{flox}. (d) Quantification of *Ar* genomic DNA within the testis was determined by qPCR analysis and reflects the AR protein loss. (e) AR nuclear staining present in the ovary of Cre-negative $AR^{flox/wt}$ and $AR^{flox/wt}$::NesCre mice. Staining is identified in granulosa cells (g) inside the basal lamina of the ovary follicles, the theca cells (t) located in the interstitium outside the

follicle basal lamina and weak staining in the oocyte nuclei (o) identified by GDF-9 staining. (f) Immunoblot and (g) quantification of ovary AR protein in $AR^{flox/wt}$::NesCre mice which is comparable to Cre-negative $AR^{flox/wt}$ level. (h) Quantification of *Ar* genomic DNA within the ovary was determined by qPCR analysis and reflects no loss in AR protein.



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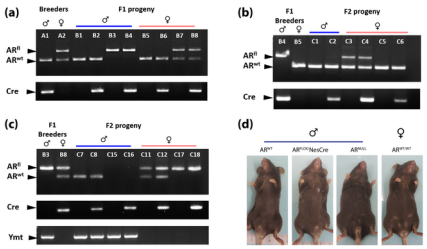

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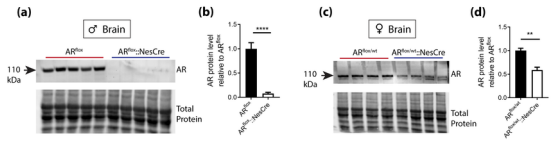
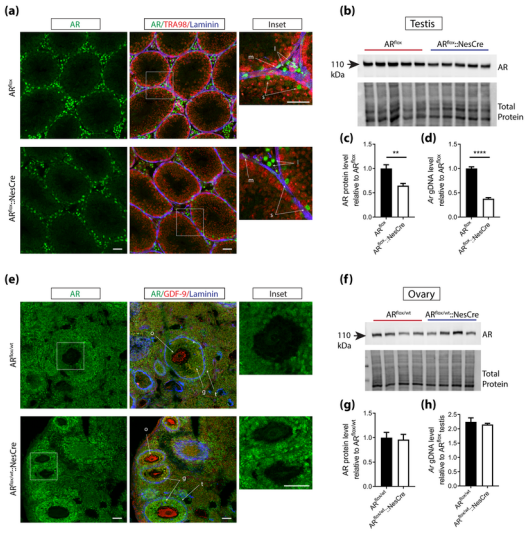


DVG_23390_McLeod_GEN_Fig3.tif



DVG_23390_McLeod_GEN_Fig4.tif

Table 1. Breeding capacity of AR^{flox}::NesCre Parent Mice

	Male (AR^{flox})	**Female ($AR^{flox\ /wt}$)**
Total Number of Breeders in Study	13	11
Total Number of Litters Produced	14	17
Total Number of Progeny	95	94
Average Litter Size	6.8	5.5
# of breeders producing no litters	3	0
Average duration to produce 1-2 litters	90 days	30 days