# The role of anti-Müllerian hormone in the brain's response to steroid hormones

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# Abstract

Many sexual dimorphisms that exist in the brain develop in the postnatal period. For males, this is when testosterone levels are minimal and anti-Müllerian hormone (AMH) is elevated. Testosterone requires the enzyme aromatase to undergo conversion to oestradiol to exert masculinising effects on the brain. Hence, the effect of AMH on aromatase was investigated.

AMH type 2 receptor (AMHR2) null mutant mice were used, with male $AMHR2^{-/-}$ mice compared to male and female $AMHR2^{+/+}$ mice. Experimental groups included perinatal, pre-pubescent and adult mice to assess different hormonal environments. In situ hybridisation was utilised to assess the presence of aromatase.

Non-radioactive in situ hybridisation was developed as a technique but remained unsuccessful following modifications.

A wide variation of aromatase expression within each genotype group was observed using radioactive in situ hybridisation. Results indicated similar levels of aromatase between the male $AMHR2^{+/+}$ and $AMHR2^{-/-}$ mice. This suggests that the role of AMH could be independent from aromatase. To confirm this, larger sample sizes will be used so statistical analysis could be performed. Location of AMH intracellular signalling molecules could be identified and genomic RNA studies could be used to assess the effect AMH has on neurons.

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# 1. Literature Review

## 1.1 Overview

Sexual dimorphisms are the differences that exist between men and women. They include variations in brain size and structure (1). Testosterone is known to mediate many of the masculinising effects on brain development, but only after conversion to oestradiol by the enzyme aromatase (2). Although testosterone has a brief elevation in the foetal and postnatal stage, it is essentially absent in childhood until puberty begins (3). Instead, the main gonadal hormone present in boys is anti-Müllerian hormone (AMH). Produced in the foetal testes, it prevents the development of the Müllerian duct which is the precursor to the female upper reproductive tract (4). This was thought to be the only male function of AMH until recently, when AMH was shown to be a hormonal regulator of male brain development (5-8) and behaviour (9, 10). These changes in brain structure and behaviour occurred when testosterone was minimal and AMH was the main gonadal hormone present. Consequently, I hypothesise that AMH modulates how the brain responds to the sex steroid hormones by influencing aromatase.

## 1.2 Brain Development

### 1.2.1 Sexual Dimorphism and Differentiation

The human brain is sexually dimorphic, with differences in structure and function. A clear difference in brain function can be seen when abnormalities occur; gender distinctions are pronounced in mental health across age groups and cultures (11). Women more commonly have disorders related to mood and anxiety, whereas men have more substance and behavioural disorders (11-14). Although social factors cannot be disregarded in the aetiology of these mental health disorders (11), the consistent gender differences suggest an underlying disparity.

Structural differences are evident since early development, with 4 year old males on average having a cerebral volume that is 9% larger than 4 year old females (1). This divergence continues throughout adolescence into adulthood (1), and a dimorphism presents in various brain areas. For example, in terms of cortical volume relative to cerebrum size (15), the right motor and somatosensory cortex (16) and Broca's area, an important area for speech production (17) are all likely to be larger in women. Men have a greater proportion of white matter in their cerebral volume (15) and a larger amygdala and hypothalamus (15). These areas that have a high degree of sexual dimorphism are also the areas with high levels of sex steroid receptors during development, indicating a possible mechanism of differentiation (15).

The historical view of neural sexual differentiation is that gonadal steroids act early in development to cause changes in anatomy, sexual behaviour and gonadotropin secretion pattern (18). A landmark paper in 1959 (19) showed that giving testosterone to female guinea pigs during a neonatal sensitive phase lead to masculinised mating behaviour that was permanent, and increased sensitivity to testosterone in adulthood. From this, the organisation/activational hypothesis of sexual differentiation was developed, which states that gonadal steroids change neural organisation during a perinatal sensitive period to alter the adult response to hormones (20, 21). In addition, this process leads to brain masculinisation for males, whereas female brain development is seen as a default pathway (18). Although developments to this hypothesis have been proposed (18), the original idea is still an important concept in sexual differentiation (22).

Another concept which ties in with sexual differentiation is the aromatisation hypothesis, proposed by Naftolin et al. in 1975 (23). Aromatase is the enzyme that synthesises local

oestrogens from circulating androgens (2). The hypothesis states that this function mediates the masculinising effects on brain development and function (2). This was first realised when newborn female rats were given testosterone in an attempt to masculinise their brains, and oestradiol was used as a control. In fact, the oestradiol turned out to be more effective (18) whereas no effect occurred when dihydrotestosterone (DHT, binds to testosterone receptors but is unable to be aromatised) was given (24). The timing of aromatase expression in rats also corresponds with its perinatal sensitive period, said to occur between gestational day 18 to postnatal day 11 (25). Aromatase mRNA expression is widespread in the rat brain (of both sexes) at gestational day 18, in parallel with serum testosterone (26, 27). This gives a chronological link between steroid release from the gonads, and the capacity for testosterone to be converted to oestradiol in the male brain. The presence of aromatase suggests that although oestrogen is typically regarded as a female hormone, in this instance it is the factor leading to normal male brain development.

### 1.2.2 Steroid Receptor Expression

Androgen and oestrogen receptors are found throughout the brain with no clear sexual dimorphisms in rats and mice (28, 29). Oestrogen receptors are commonly found at high levels in areas related to gonadotropin release and sexual behaviours while androgen receptors are found in motor control regions and cranial nerve nuclei (28, 30). These areas containing high numbers of oestrogen receptors are regions commonly studied for evidence of sexual dimorphism in the mouse brain. For example, the bed nucleus of the stria terminalis (BNST) has a sexual dimorphism in its cell number and volume by 9 days old (31), increasing to 35% greater in males than females by adulthood (32).

The absence of sexual dimorphism in receptor distribution, but a sexual dimorphism in brain structure indicates that the structural differences could depend on factors other than oestrogens and androgens. Commonly stated hypotheses are alterations in steroid receptor binding, transcription factors activated by such binding, or an interaction between the ratio of androgen to oestrogen receptor binding. There is also the intriguing new proposition that other gonadal hormones may be involved (6-9).

### 1.2.3 Oestrogen in the Brain

Oestrogens are present in females in three forms: oestrone, oestradiol and oestriol. At reproductive age, oestradiol (the most potent) is the major occurring oestrogen (33). However, early female development occurs in a low oestrogen environment with the ovaries

being non-steroidal until puberty. Any oestrogen present in a female foetus will be from the adrenal glands, the mother or male littermates (34).

As male-typical brains develop in the presence of oestrogens (according to the aromatisation hypothesis), this leads to the idea that female-typical brains develop in an oestrogen free environment. Alpha-fetoprotein (AFP) fulfils this role by actively protecting the female brain from adrenal or external oestrogens from the mother or male littermates (35). AFP is a plasma protein produced in the foetus which readily binds circulating oestradiol in rats and mice (35). Female mice that do not express AFP have male-like brain anatomy, abnormal sexual behaviour and infertility (34) which is rescued by aromatase inhibition during gestation (36). This shows that the presence of oestrogen without its binding protein in a developing female leads to some male-typical features, while the absence of oestrogen permits normal female development.

To expand on this, oestradiol has been demonstrated to have direct masculinising effects on the developing female brain. Female mice given oestradiol on the day of birth have male-like numbers of cells in the BNST (32). Using oestrogen receptor agonists also increased cell numbers, but not to the same level as oestradiol (32). Oestradiol administered to neonatal female mice also lead to the development of male-like numbers and projection pattern of aromatase expressing neurons (37). Here we see evidence of a classically ‘female’ hormone causing typical male dimorphisms. In addition to anatomical changes, the oestradiol treated newborn female mice also have altered behaviour. They had male-like territorial behaviour and urine deposition pattern (37) while they had suppressed female sexual behaviours, actively rejecting mounts (37, 38). Overall, the presence of oestradiol in the perinatal phase for female mice appears to support the development of many male-typical characteristics.

However, the idea that female sexual differentiation occurs in the absence of sex hormones has been challenged by Bakker and Baum. Their hypothesis states that after birth, oestradiol acts as a feminising agent on female brains, when α-fetoprotein expression is decreasing, and the ovaries are starting to function and produce oestrogens (34). This was supported by a study that showed female aromatase knockout mice in adulthood had deficits in reproductive behaviours such as odour preference and lordosis behaviour, indicating oestradiol was needed in some capacity for normal female behaviour to develop (39). This dual action hypothesis for oestradiol is not mutually exclusive with the aromatisation

hypothesis, the former acting between birth and puberty and the latter acting during prenatal development. It has been shown that for the BNST, oestradiol has masculinising effects perinatally, and demasculinising effects during puberty (40). This is a fascinating concept of a chronological switch for oestrogen, acting initially to produce male-typical development and then mediating normal female function.

### 1.2.4 Testosterone in the Brain

The role of testosterone in brain development is complex, as it can act via androgen receptors, or after conversion to oestradiol via oestrogen receptors (41). Castrated animals given testosterone supplementation are commonly studied to investigate the effects on brain structures or behaviour (19, 42-45). However, interpretation of these studies can be problematic; it is unclear whether the results are from the action of testosterone or following its conversion to oestrogen. An alternate study design is to use dihydrotestosterone as it is unable to be aromatised, restricting its effects to the androgen receptors only (24, 46). The use of selective aromatase inhibitors also allows for endogenous androgenic effects, but prevents any new formation of oestradiol (47). The two latter experimental designs also have the benefit of not affecting the actions of other endocrine products from the testes.

The castration method was proved inadequate when the sexual dimorphism in the posterodorsal medial amygdala (MePD) was investigated. The MePD is 65% larger in adult male than female rats, with castrated males having volumes equal to those of females (42). Females and castrated males given testosterone supplementation had similar MePD volumes to normal males (42), but when this was repeated with separate treatment groups of oestradiol and dihydrotestosterone, only oestradiol had an effect on MePD volume (48). This shows that the initial discovery of testosterone being responsible for the difference in MePD volume was incorrect, and in truth it was the action of oestradiol.

The use of DHT in testosterone related studies also allowed for investigation into whether testosterone acts on androgen or oestrogen receptors to facilitate male brain development. Female rats given DHT in utero and postnatally were compared against non-treated females at adulthood. There was little difference in their ovarian morphology and sexual behaviour (24). In addition, when newborn rats were given either DHT or testosterone, the DHT had no effect on lordosis (female sexual receptivity) behaviour. Testosterone inhibited lordosis behaviour almost completely (49). This indicates that the testosterone inhibition was likely due to conversion to oestradiol, as the androgen DHT had no effect on female-typical sexual

behaviour. The identification of the exact steroid hormone that is involved in these behaviours is a definite advantage for studies using this selection of testosterone, oestradiol and DHT. The lack of this choice in many earlier studies makes their results difficult to construe. The results from castration studies are also confounded by the fact that testes do not solely secrete testosterone. Inhibin B and AMH for example, are also secreted (50).

## 1.3 AMH

### 1.3.1 AMH and Sexual Development

AMH is a member of the Transforming Growth Factor β (TGFβ) superfamily of growth and differentiation factors (51). It is another gonadal hormone that acts to facilitate male reproductive development. It initiates its response in cells by binding to its Type II receptor, which incorporates the Type 1 receptor. Phosphorylation of intracellular signalling molecules Smad 1, 5 or 8 occur, which translocate to the nucleus to regulate gene transcription (52, 53). The gonads in the embryo remain undifferentiated up to the 7th week of gestation, at which AMH begins to be expressed from the Sertoli cells of the testes (4). AMH acts to cause the Müllerian duct (the precursor to the female upper reproductive tract) to regress by the $8^{th}$ and $9^{th}$ week of gestation, via apoptosis and epithelial-mesenchymal transition (4).

The regression of the Müllerian duct in males is essential to a functional reproductive tract. $AMH^{-/-}$ male mice develop as pseudohermaphrodites, with both male and female reproductive tracts superimposed on each other (54). Although they can still make viable sperm it is anatomically blocked from exiting the male normally, regurgitating into their own hypoplastic uterus and seminal vesicles (54). Female mice over-expressing AMH have no uterus nor uterine tubes, a blind vagina and no ovaries by adulthood (55). Therefore, the presence of AMH in males and the absence of it in females is required for fertility.

### 1.3.2 AMH Levels

AMH is the major gonadal hormone produced by boys until puberty. At birth, levels are comparatively low (~150pmol/L) in human boys (56). After a brief rise at 6 months, levels are maintained throughout childhood (~700pmol/L), declining only slightly with age. (56). A sudden decrease occurs once they begin puberty and by the time boys reach their middle stage of pubertal development, their levels are reduced to adult male levels (56). Here they start to overlap with female levels, which after being minimal at birth have had a minor rise throughout childhood and puberty (57). Prior to puberty, AMH levels are at least 10 times higher in boys than girls (57). There is currently no explanation for why it should be raised in boys for such a long time as its function in regressing the Müllerian duct has clearly finished.

Concurrently, as AMH is declining in puberty in boys, testosterone is rising (56) (Figure 1.3.2.1). Following a ‘mini puberty’ at 1-4 weeks of age, where testosterone has a brief

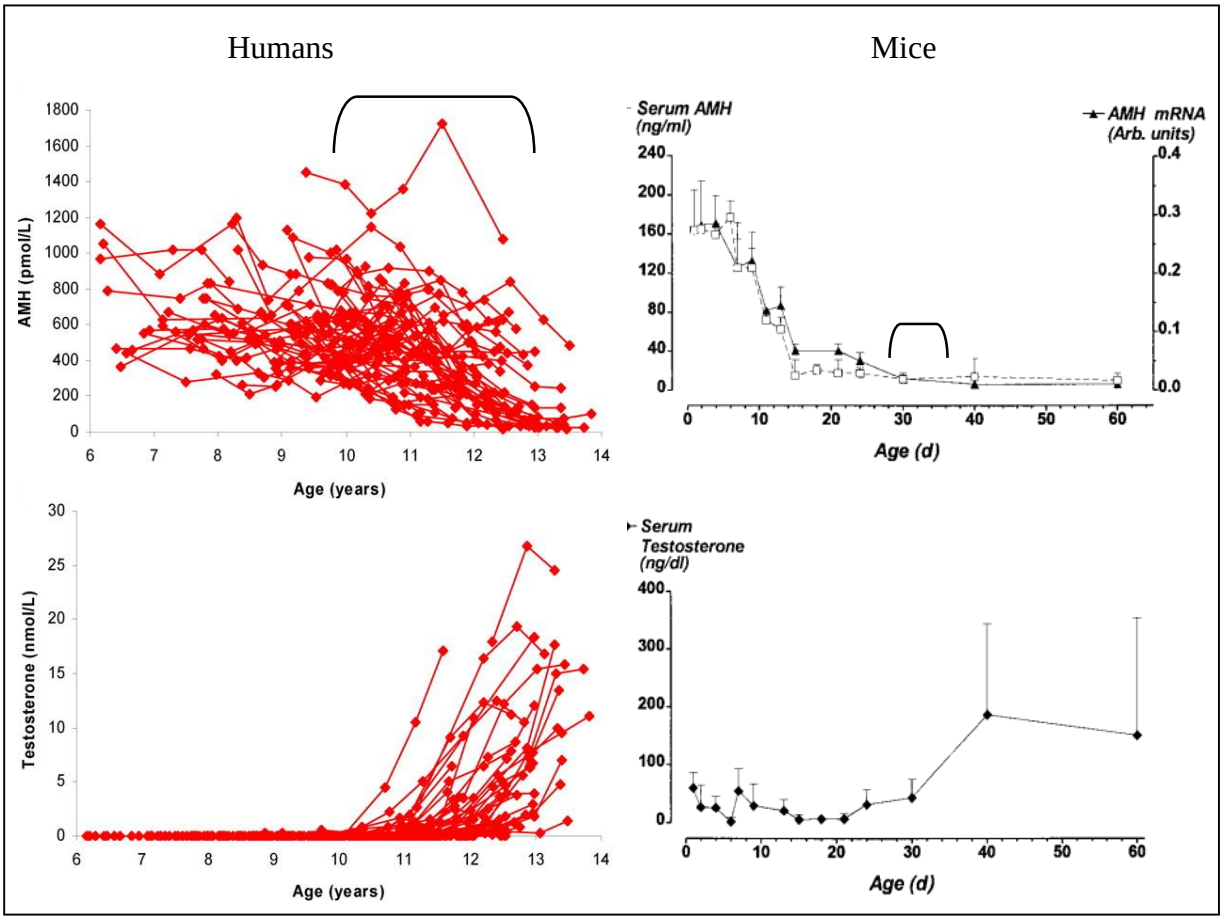


**Figure 1.3.2.1: Serum AMH and Testosterone in human boys and mice. Measurements of serum AMH and testosterone in human boys and mice at chronological ages, adapted from Al-Attar et al and Aksglaede et al (30, 56). The black brackets show the approximate start of puberty (58, 59). There is a gradual decrease of AMH as puberty is reached, and a distinct increase in testosterone. The main gonadal hormone present prior to puberty is AMH.**

surge (8nmol/L), testosterone levels stay low throughout childhood (below 1nmol/l) (3). As puberty begins, the levels begin to rise and reach a mean of 17nmol/L in 16 year old boys. This pattern shows that in boys, AMH is high and testosterone is low, and after puberty the reverse occurs. This makes AMH the predominant gonadal hormone present in boys in their early development. For girls, both AMH and testosterone are low throughout childhood and puberty (3). Therefore AMH is sexually dimorphic.

Mouse models have shown a similar pattern, although they have their 'mini puberty' at the time of birth (60). Instead of AMH levels increasing during the first year of life, male mice have elevated levels to 6 days of age, which decreases significantly from day 7 to day 13 (30). As it is in humans, when puberty is underway in the mouse AMH levels are low and testosterone levels are high.

### 1.3.3 AMH and Brain Development

The function of AMH regressing the Müllerian duct occurs early in development, before the sustained levels in childhood. Extensive brain development occurs when AMH is the main testicular hormone present and testosterone is minimal (Figure 1.3.3.1) (61). This presents the idea that AMH is a contributing factor to development of the brain.

Sexual dimorphisms in the mouse brain have been identified in periods where AMH is high and testosterone is low. This can be observed in the bed nucleus of the stria terminalis (BNST) and the sexually dimorphic nucleus of the preoptic area (SDN). Pre-pubescent wild type male mice typically have 25% more neurons in their BNST compared to wild type female mice (puberty generally occurs after 30 days (58)). At adulthood this difference increases due to a combination of atrophy in the females and growth in the males, to the extent that adult males have double the number of neurons in the BNST compared to the adult females. However, the pre-pubescent $AMH^{-/-}$ male mice had similar numbers of BNST neurons to females. During puberty, the $AMH^{-/-}$ males underwent the male-typical increases of neurons (this appeared to be AMH independent) so at adulthood they no longer had female-like neuron numbers (Figure 1.3.3.2). But this growth did not completely correct the pre-pubertal difference in neurons between the knockout and wild type males. Consequently at adulthood, the $AMH^{-/-}$ male mice still had a significant reduction of neurons compared to the wild type male mice (6, 8). In addition, male $AMH^{-/-}$ mice have female-like cerebella in terms of total volume, cell number and Purkinje cell size (7). These differences in cell number and size between the male wild type and knockout mice have occurred following the

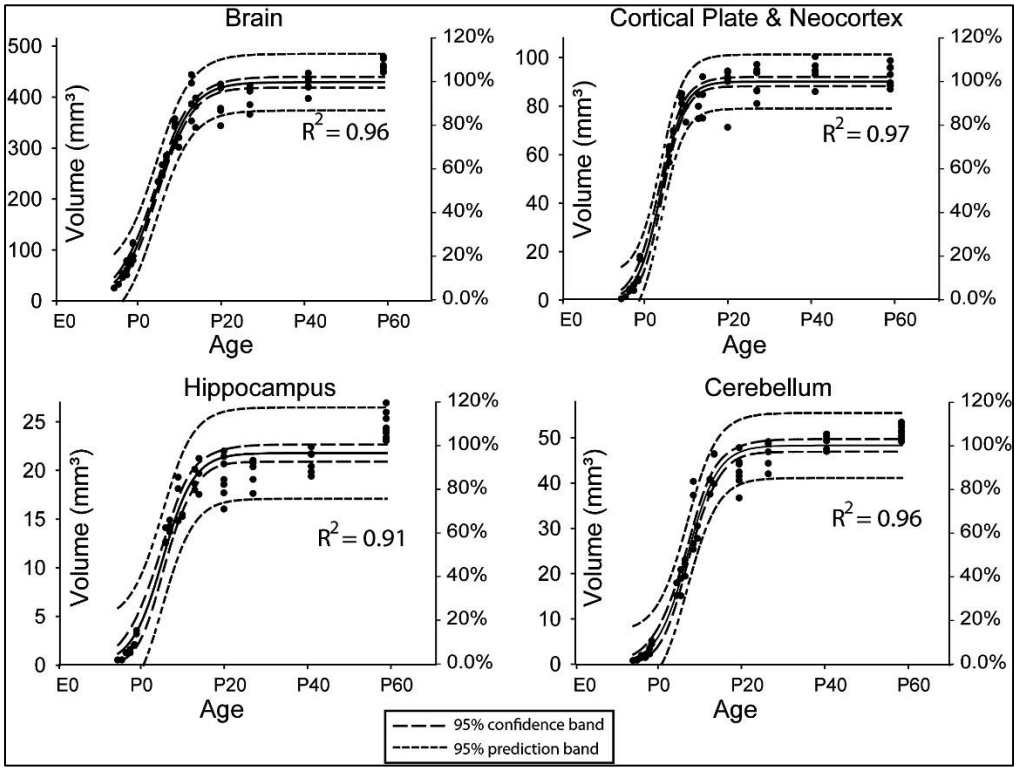


**Figure 1.3.3.1: Volumes of the mouse whole brain, neocortex, hippocampus and cerebellum compared to estimated adult volumes at chronological ages, from Chuang et al (56).There is a dramatic increase in volumes in all areas shown prior to 20 days (before puberty occurs). This is when AMH is the main gonadal hormone present and the testosterone rise has not occurred.**

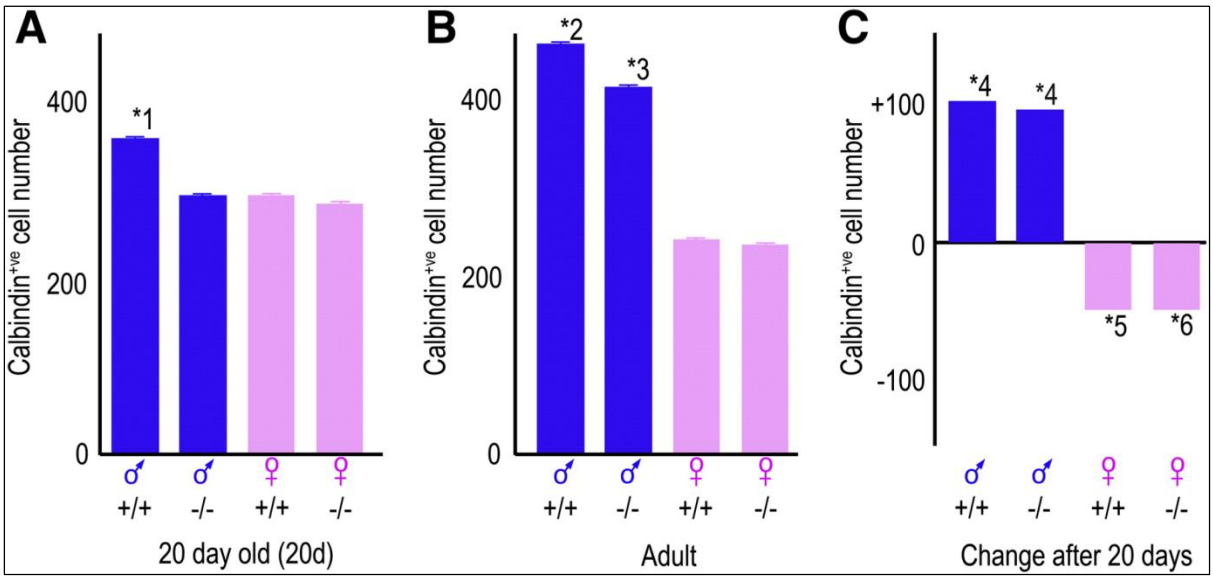


**Figure 1.3.3.2: Calbindin cell number in the SDN in AMH$^{-/-}$ and Wild type Mice, from Wittmann and Mclennan (6). At 20 days old the AMH$^{-/-}$ males have female-like cell numbers. However, after puberty they do not undergo the female reduction in cell number.**

same foetal testosterone surge, so the main difference in their hormonal milieu during development is the presence of AMH. From these results we can reason that AMH appears to be an important factor in the development of sexual dimorphism in the brain.

### 1.3.4 AMH and Sexually Dimorphic Behaviour

A number of behaviours have been found to be different in $AMH^{-/-}$ mice compared to their wild type counterparts. Male-typical exploratory behaviours can be quantified in novelty preference and rearing in field tests (9, 62). Male $AMH^{-/-}$ mice at 20 days old were found to have female-like preferences for novel or familiar objects (10), and also female levels of rearing (9).

Behaviours more directly related to sexual function were also tested. Olfactory preference for male and female pheromones was tested using soiled bedding. All the experimental groups (adult and pre-pubertal male and female, wild type and knockout) preferred those belonging to the opposite sex. All groups except the male $AMH^{-/-}$ mice preferred to sniff the soiled bedding over the clean bedding (8). The preferential sniffing of female-soiled, rather than male-soiled bedding shows that that the male $AMH^{-/-}$ mice do have an interest in females and have a functioning olfactory system, but appear to be disinterested at the same time as they sniff more clean bedding. Similar to the differences in brain structures, these differences in behaviours were found prior to the pubertal testosterone surge. Given the male knockout and wild type mice have had the same foetal testosterone peak, this indicates a possible role for AMH in development of this behaviour. One possible mechanism is that AMH influences the actions of how testosterone acts on the male brain. Testosterone requires aromatase to undergo conversion to oestradiol to produce male-typical effects. It is therefore possible that aromatase is a factor influenced by AMH that leads to these changes in behaviour.

## 1.4 Aromatase

### 1.4.1 Aromatase Expression

Aromatase is found in discrete regions of the brain. Studies on adult rodent brains have revealed a distinctive pattern of aromatase activity, particularly around the hypothalamus and amygdala which are involved in reproductive behaviour and function (63). Aromatase activity was higher in males than females in almost all areas, with differences reaching 70% lower for females in the bed nucleus of the stria terminalis (BNST) (64).

These areas of high aromatase expression also correlate with areas of high aromatase activity, such as the medial preoptic nucleus, BNST and the cortical and medial amygdala (64). These are areas involved in reproductive function and behaviour (63), with the BNST and medial amygdala in particular being shown to regulate sexual and aggressive behaviours (37). In addition, these areas high in aromatase overlap extensively with areas high in oestrogen receptors. This suggests that a local effect of the aromatase is occurring, where its oestradiol product can act on the same (or nearby) cells (65).

In addition to sexual differences, there are transient differences in aromatase expression and activity during development. Activity and expression can be detected in an embryonic rat brain at day 16 (26, 27) localised to the preoptic area (26). Levels increase at embryonic days 18 and 20, with expression now occurring in the more typical areas of the hypothalamus, BNST and medial amygdala (26). Aromatase levels in males and females are similar prior to birth but by postnatal day 2 aromatase levels decrease and a sexual dimorphism is present (66). This identifies the possible age where the presence of aromatase may be essential for normal development. After postnatal day 2, male rats have significantly higher levels in the BNST and sexually dimorphic nucleus of the preoptic area (SDN), while females have higher levels in the medial preoptic nucleus. By postnatal day 6, many of these sex differences are no longer significant, but remain so in the BNST (66). This suggests that by 6 days of age, aromatase may have already influenced the sexual dimorphic structure in those areas. This gives us an indication of what time points could be valuable in examining the functions of aromatase.

**Table 1.4.1.1: Aromatase mRNA Levels in the adult rat brain**

| Level of Aromatase mRNA | High | Reference | Intermediate | Reference |
|---|---|---|---|---|
| Hypothalamic structures | • Medial Preoptic Nucleus | (65, 67, 68) | • Ventromedial nucleus<br>• Strial Preoptic Area | (65)<br>(67) |
| Limbic structures | • Bed nucleus of the stria terminalis<br>• Medial amygdala | (65-67)<br>(65-68) | • Cortical amygdala<br>• Hippocampus | (65, 67)<br>(67, 68) |

### 1.4.2. Aromatase and Brain Sexual Differentiation

As normal brain development requires aromatase to synthesise local oestrogens from circulating androgens (2) this places aromatase at high importance in sexual differentiation of the brain. There are multiple sexually dimorphic areas in rodents that contain high levels of aromatase (69), often in areas related to reproductive behaviours. One of these areas in the rat brain is the Sexually Dimorphic Nucleus (SDN), located in the medial preoptic area of the hypothalamus which is known for sexual function and behaviour (26, 63).

The SDN is 2-3 times larger in male than female rats and appears to be dependent on aromatised testosterone (70) present during the perinatal stage (71). Perinatal administration of testosterone or oestrogens to female rats increased their SDN volumes (43, 71). To identify whether testosterone was exerting an effect only after conversion to oestradiol or in addition to acting as an androgen, antagonist based studies were performed. Oestrogen antagonists (72) and aromatase inhibitors (73) were found to reduce the SDN volume of male rats to female-like volumes, while an androgen antagonist had no effect (72). This local oestrogen synthesis by aromatase is therefore crucial in the development of male-typical sexual dimorphisms in the brain.

### 1.4.3 Aromatase and Sexual Behaviour

The presence of aromatase in neural pathways for reproductive behaviour (37) gives an indication to its role in regulation sexual behaviour (63). In particular, it has been shown to be involved in some of the sexual behaviours that were found to be different in $AMH^{-/-}$ mice. Akin to the male $AMH^{-/-}$ mouse, the male aromatase knockout mouse appears to simultaneously prefer female odours, but also displays a significant level of disinterest for the pheromones of both male and female mice (74). Other changes in sexual behaviour seen in the aromatase knockout mouse include decreases in mounting, intromission and ejaculation (74-77). Similar results were found for male rats given an aromatase inhibitor perinatally (73, 78-81) and they also displayed a sexual preference for males over oestrous females (73, 78, 82, 83) . For most of these studies the critical time for aromatase to be inhibited was the early postnatal stage as the rats that were given aromatase inhibitor on those days had more significant differences compared to controls, and compared to rats that were only given the inhibitor while still in gestation. This is in agreement with the high expression of aromatase during the early postnatal stage, and gives us an indication of when the presence of aromatase is essential in development of normal male sexual behaviour.

### 1.4.3 Aromatase Regulation

Aromatase itself is regulated through an androgen receptor-mediated feed-forward mechanism with testosterone being both the substrate and regulator (84). However, testosterone itself has wide ranging actions at each age group and brain regions, so aromatase regulation can become complex (63).

Androgen regulation during the foetal stage is unclear (85). Hypothalamic explants from prenatal rats failed to increase their aromatase activity when cultured with sex steroids (86). In addition, castration of foetal monkeys resulted in no change in brain aromatase activity, with and without androgen treatment (87). There is some evidence to show that in adult rats, aromatase is regulated by androgens. Castrated male and female adult rats showed a reduction in aromatase activity and mRNA in the hypothalamic and preoptic area (HPOA) (68). This effect was rescued by testosterone and dihydrotestosterone treatment, but not oestradiol, implicating an androgen mediated mechanism (68). However, the castration method of these studies means that the contribution of the other gonadal hormones cannot be examined.

Nevertheless, the role of androgens has also been demonstrated by other means. Adult male rats deficient in androgen receptors have significantly reduced brain aromatase activity, unaltered by castration or testosterone treatment (88). Reduced brain aromatase is also seen in male rats treated with an androgen antagonist compared to controls (89). Overall, testosterone and dihydrotestosterone stimulate aromatase activity (68) while the absence of androgen receptors (88) or administration of an antagonist (89) decreases aromatase activity, concluding that androgens are a regulator of aromatase in the adult rat brain. This means that the difference in basal circulating androgen levels in male and female rats also leads to a difference in aromatase levels (84). But male rats also have a greater response to androgen stimulation, shown by castrated males having higher aromatase activity and mRNA following a dose of DHT compared to ovariectomised females (90).

Although testosterone has been extensively shown to regulate aromatase in the brain, AMH has been shown to have some effects on aromatase in the ovary. Initially, this was discovered when ovaries cultured in AMH developed testes-like structures and increased their testosterone secretion (91, 92). This has been found in the ovaries of various species (92, 93) but conflicting results have been found in testicular tissue (93, 94). The actions of AMH upon other systems have not been thoroughly investigated as of yet.

## 1.5 Study Proposal

The aims of this study will be to determine if the levels of aromatase are different in aromatase knockout mice compared to their wild type counterparts.

To investigate this, three genotypes will be used: male and female wild type mice ($AMHR2^{+/+}$), and male AMH receptor knockout mice ($AMHR2^{-/-}$). This will allow us to compare if the male $AMHR2^{-/-}$ mice have levels of aromatase more similar to wild type females, or males. Three age groups will be used, 2 day old mice, 20 day old mice and 42 day old mice. This allows us to look at different hormonal environments. For wild type males at 2 days old, there is high testosterone, high AMH, high aromatase levels comparatively. Pre-pubescent 20 day old mice have low testosterone, decreasing AMH and low aromatase. Adult 42 day old mice have high testosterone, low AMH and low aromatase. Female mice at all stages have low testosterone and low AMH, and lower aromatase levels compared to males.

Experimental techniques used will include non-radioactive and radioactive in situ hybridisation for the aromatase enzyme. Immunohistochemistry will be used for intracellular AMH signalling molecules Smad 1, 5 and 8.

# 2. Methods

## 2.1 Materials

### 2.1.1 Animal tissue collection

**2% Paraformaldehyde**

100 ml $dH_2O$ and 4 g paraformaldehyde heated to 50°C

Add 1M NaOH drop wise until clear and make up to 200 ml with 0.2M PB.

Filter and adjust pH to 7.4

**30% sucrose**

30 g sucrose

Make up to 100 ml with distilled $H_2O$

### 2.1.2 In situ hybridisation stock solutions

**Diethylpyrocarbonate (DEPC) treated water**

1 ml DEPC

2000 ml $dH_2O$

Stir overnight and autoclave for 40 minutes to inactivate the DEPC

**2M Tris**

121.14 g Tris

400 ml $dH_2O$

Adjust pH to 8, make up to 500 ml with $dH_2O$, and autoclave for 20 minutes

**TE Buffer**

1 ml 2M Tris

400 µl 0.5M EDTA ph8

Make up to 200 ml with $dH_2O$ and autoclave for 20 minutes

**3M Sodium acetate**

24.6 sodium acetate anhydrous

80 ml $dH_2O$

Adjust pH to 7.5 with glacial acetic acid, make up to 100 ml with $dH_2O$ and autoclave for 20 minutes

**0.2M Phosphate Buffer**

160 ml 0.5M $Na_2HPO_4.2H_2O$

40 ml 0.5M $NaH_2PO_4.H_2O$

Adjust pH to 7.3 and make up to 500 ml with $dH_2O$

**20 x SSC**

175.3 g NaCl

88.2 g trisodium citrate.$2H_2O$

800 ml $dH_2O$

Adjust pH to 7 and make up to 1 L with $dH_2O$ and autoclave for 20 minutes

**5 x TEA**

92.8 g TEA HCl

45 g NaCl

900 ml $dH_2O$

Adjust pH to 8 and make up to 1 L with $dH_2O$

**tRNA stock solution**

50 mg tRNA

1 ml DEPC water

Freeze in aliquots at -20°C

**Hybridisation Buffer**

7.5 g dextran sulphate

10 ml DEPC water

Heat to 68°C until dissolved. Cool to RT and make up volume to 15 ml with DEPC water.

To 10 ml of this solution, add

5 ml 100% formamide

1 ml 3M NaCl

100 µl 0.5M EDTA pH8

200 µl Denhardt's solution

Store at -20°C until use, when 154.4 mg DTT is added

**Fixer for slide development**

240 g sodium thiosulphate

600 ml tap water

Stir to dissolve, then make up to 800 ml

### 2.1.3 In situ hybridisation working solutions

These are solutions made up on the day of use, sufficient for 75 slides.

**2% Paraformaldehyde**

300 ml $dH_2O$ heated to 45°C

Add 12 g paraformaldehyde and heat up to 65°C

Add 1M NaOH drop wise until clear and make up to 600 ml with 0.2M PB.

Filter and adjust pH to 7.4

**0.5 X SSC**

30 ml 20 x SSC

1170 ml DEPC water

**RNase Buffer**

166 ml 3M NaCl

5 ml 2M Tris

Make up to 1 L with DEPC water

**Proteinase K Solution**

600 ml RNase Buffer

120 µl proteinase K, add just before use

**1 x TEA**

120 ml 5 x TEA

480 ml DEPC water

**1 x TEA with acetic anhydride**

120 ml 5 x TEA

480 ml DEPC water

1.5 ml acetic anhydride, add just before use

**2 x SSC**

150 ml 20 x SSC

Make up to 1.5 L with DEPC water

**Box Buffer**

18 ml 20 x SSC

45 ml formamide

27 ml DEPC water

**50% ethanol**

125 ml 100% ethanol

125 ml DEPC water

**80% ethanol**

200 ml 100% ethanol

50 ml DEPC water

**95% ethanol**

237.5 ml 100% ethanol

50 ml DEPC water

**2 x SSC (post hybridisation)**

300 ml 20 x SSC

Make up to 3 L with $dH_2O$

If using radioactive labelled probes, include:

6 ml 0.5M EDTA

2103 µl β-mercaptoethanol

**0.1 x SSC (post hybridisation)**

30 ml 20 x SSC

Make up to 3 L with $dH_2O$

If using radioactive labelled probes, include:

6 ml 0.5M EDTA

2103 µl β-mercaptoethanol

**0.5 x SSC (post hybridisation)**

30 ml 20 x SSC

Make up to 1.2 L with $dH_2O$

**RNase A Solution**

600 ml RNase Buffer

1.2 ml RNase A, add just before use

**Buffer 1**

150 ml 2M Tris

150 ml 3M NaCl

2.5 L $dH_2O$

Adjust pH to 7.5 and make up to 3 L with $dH_2O$

**Buffer 3**

75 ml 2M Tris

49.5 ml 3M NaCl

15.2475 g $MgCl_2.6H_2O$

1.4 L $dH_2O$

Adjust pH to 9.5 and make up to 1.5 L with $dH_2O$

**1 x SSPE**

125 ml 20 x saline-sodium phosphate-EDTA

Make up to 2.5 L with $dH_2O$

**Fixer**

240 g sodium thiosulfate

600 ml tap water

Stir to dissolve, then make up to 800 ml with tap water

### 2.1.4 Immunohistochemistry solutions

**0.01M Phosphate Buffer (PB)**

12.1 g $Na_2HPO_4.2H_2O$

4.06 g $NaH_2PO_4.2H_2O$

Dissolve in 5L $dH_2O$, make up to 10 L and adjust pH to 7.2-7.4

**Glycine in 0.01M Phosphate Buffer**

3.8 g glycine

500 ml 0.01M PB

**Primary Antibody Diluting Buffer**

0.4 g NaCl

20 µL Polysorbate 80

10 ml 0.01M PB

100 mg Bovine serum albumin (Jackson Immunologicals, Pennsylvania, USA)

**Secondary Antibody Diluting Buffer**

10 ml 0.1M PB

100 mg Bovine serum albumin (Jackson Immunologicals, Pennsylvania, USA)

**Wash Buffer**

20 g NaCl

1 ml Polysorbate 80

1 L 0.01M PB

**Hydrogen Peroxide 15%**

1 tablet $H_2O_2$ urea

1 ml $dH_2O$

**Hydrogen Peroxide 1%**

67 µl $H_2O_2$ 15%

933 µl 0.01M PB

## 2.2 Animal tissue

Transgenic mice with a non-functioning AMH receptor 2 (AMHR2) were used (95). $AMHR2^{-/-}$ males and $AMHR2^{+/+}$ males and females were bred from AMHR2 heterozygous crosses. Mice had ad libitum access to water and food pellets. The room was kept at 21 ± 2°C and 45% humidity with a 12:12 hour light/dark cycle.

Two mice were analysed for each genotype at each age group, except the male mice for the 2 day old group, for which there were 3 mice each. This gave a total of 20 mice. Each mouse was anaesthetized with ketamine (225mg/kg) and medetomidine (3mg/kg). Transcardial perfusion using a peristaltic pump was performed for 3 minutes using 2% paraformaldehyde in 0.1 M phosphate buffer (2 day mice 0.5 ml/min, 20 day mice 5 ml/min, 42 day mice 13.3 ml/min). Brains were dissected from the mice and left in 2% PFA for post-fixation for 3 hours, and then in 30% sucrose overnight at 4°C for cryoprotection. Brains were frozen in frozen section media (FSC 22, Leica Biosystems, Victoria, Australia) on dry ice and kept at -80°C.

## 2.3 In situ hybridisation

In situ hybridisation is a technique used to identify the location of a specific nucleic acid sequence (RNA or DNA) in a tissue section and determine the level of gene expression. The underlying principle is that the gene sequence of interest can be targeted by using complementary probes of RNA or DNA which will hybridise with it. These hybridised probes are labelled (fluorescence, radioactive labelling, or an antigen) so that they can be visualised.

In situ hybridisation relies on the presence of the mRNA of interest so extensive measures must be taken to ensure that all steps leading up to hybridisation are as free of ribonucleases (RNase) as possible. The cryostat was cleaned with 70% ethanol and all equipment for tissue cutting was cleaned with RNase Away (Life Technologies, California, USA) before each use. All solutions for the procedure were made up in RNase free conditions, using diethyl pyrocarbonate (DEPC) treated water, and with all glassware baked at 200°C for at least 4 hours. The protocol up to and including the hybridisation step was performed in a specialised RNase free fumehood and associated equipment.

The brain area containing the bed nucleus of the stria terminalis (BNST) was sectioned at a thickness of 16µm in a cryostat set at -25°C to -23°C. Due to the increasing sizes of the brains, 36 sections were obtained from 2 day mice, 42 sections from 20 day mice and 60 sections from 42 day mice. Serial sections were taken, alternating between sections being taken for immunohistochemistry, non-radioactive in situ hybridisation, and radioactive in situ hybridisation. Brains from 20 day mice and 42 day mice were float mounted in $dH_2O$ placed in the cryostat onto Superfrost Plus slides (LabServ, Thermo Fisher Scientific, Massachusetts, USA), and dried at room temperature (RT) for 1.5 hours. Brains from the 2 day mice were thaw mounted (their small size did not permit float mounting) onto Superfrost Plus slides, and dried at RT for 30 minutes. Slides from all tissues were kept at -20°C until use. Storage varied from 1 to 3 months.

## 2.4 Aromatase non-radioactive in situ hybridisation

### 2.4.1 Probe identification

Using the Primer Blast tool from NIH, primer pairs were identified in the mouse CYP19 (Aromatase) gene sequence. Possible primer pairs were chosen from the selection based on having less than 3 consecutive cytosines (C) or guanines (G) at the 3' end which would affect binding as the C-G bond is stronger than the adenosine-tyrosine bond. Primers also had to cross an exon, and not be specific to any other major transcripts.

Characteristics of the chosen primers were checked with the IDT Oligo Analyzer. Hairpins were checked for their temperature being less than annealing temperature, their ΔG being preferably between 0-2 and that less than 4-5 base pairs were matching on the hairpin. Self-dimers and hetero-dimers had to have a ΔG less than 25% of the maximum ΔG, up to 3-5-base pairs matching, and no 3' end binding to each other over 2 base pairs. From these selection criteria, 2 possible primers were identified and were ordered. A SP6 tag (AATTAGGTGACACTATAGAATAG) was added to the forward primer for the antisense probe and a T7 tag (TAATACGACTCACTATAGGGAGA) was added to the reverse primer for the sense probe.

### 2.4.2 RNA extraction

Ovaries from knockout and transgenic mice for Brain AMH were used for RNA extraction using the Acid guanidinium thiocyanate-phenol-chloroform extraction method (Trizole). The acidic solution separates RNA from the DNA and proteins.

A mortar and pestle was cooled down using liquid nitrogen, into which the ovaries were placed and ground down. The powdered ovaries were put into a tube with 0.5 ml Trizole and mixed with a homogeniser. The tube was shaken by hand for 15 seconds with 0.1 ml chloroform, and then left at RT for 10 minutes. The tube was then spun in the centrifuge at 4°C and 12000G for 5 minutes. At this point, the contents in the tube should be layered with proteins at the bottom, DNA in the middle and RNA at the top. The RNA layer was taken off by pipette, mixed with 250 µl propanol, and left overnight at -20°C. The next morning, it was spun in the centrifuge at 4°C and 12000G, this time for 10 minutes. Supernatant was pipetted off, leaving a pellet of the RNA on the bottom. Added to this pellet was 1 ml of 75% ethanol, then it was briefly vortexed and centrifuged at 4°C and 13000G for 5 minutes. As much supernatant was taken off as possible, the rest was evaporated by placing it in a warming plate at 65°C. The pellet was resuspended in 30 µl of sterile water.

**Table 2.4.1.1 Primer sequences**

| | Forward 5’ to 3’ | Reverser 5’ to 3’ |
|---|---|---|
| Primer 1 | CGTGGATGTGTTGACCCTCA | CTCCACGTCTCTCAGCGAAA |
| Primer 2 | GAGGTCGAAGCAGCAATCCT | AGGGAAGTACTCGAGCCTGT |

The RNA was quantified against standard solutions on the microvolume spectrophotometer. Only one of the ovaries gave any substantial amount of RNA, at 103.5ng/µl. The RNA was treated to remove any DNase present using the Turbo DNase Kit (Life Technologies, California, USA) used according to manufacturer's instructions. The RNA was stored at -80°C.

### 2.4.2 cDNA synthesis

cDNA was synthesised from the extracted RNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, California, USA). Added to 5 µl of the RNA was 9 µl $H_2O$, 4 µl VILO reaction mix and 2 µl enzyme mix. This mixture was left at RT for 10 minutes, put in a 42°C heating block for 60 minutes, 85°C for 5 minutes and then stored at -20°C.

### 2.4.3 Polymerase Chain Reaction (PCR)

The cDNA synthesised was then used to make the probe template. A PCR reaction was set up with 12.5 µl master mix (2X Reddy Mix, Thermo Fisher Scientific, Massachusetts, USA), 9.5 µl $H_2O$, 0.5 µl each of the forward and reverse primers, and 2 µl cDNA. Control tubes used $H_2O$ instead of the cDNA. This went in a cycle of:

1) 94°C for 2 minutes
2) 94°C for 30 seconds
3) 58°C for 1 minutes
4) 72°C for 45 seconds
5) Repeat steps 2-4 30 times
6) 72°C for 5 minutes
7) End at 10°C

This resulted in a product for the second primer set, which was 247 base pairs. This was confirmed by a gel electrophoresis (Figure 2.4.4.1). The successful PCR protocol was repeated with 5 tubes, and the products were pooled together and kept at -20°C. The probe created from this PCR product is named Probe A. Purification of the PCR product was performed using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The product was stored on ice for 5 minutes, and then the concentration was checked on the microvolume spectrophotometer. This gave a mean of 69.81 ng/µl which was used for the DIG labelling process.

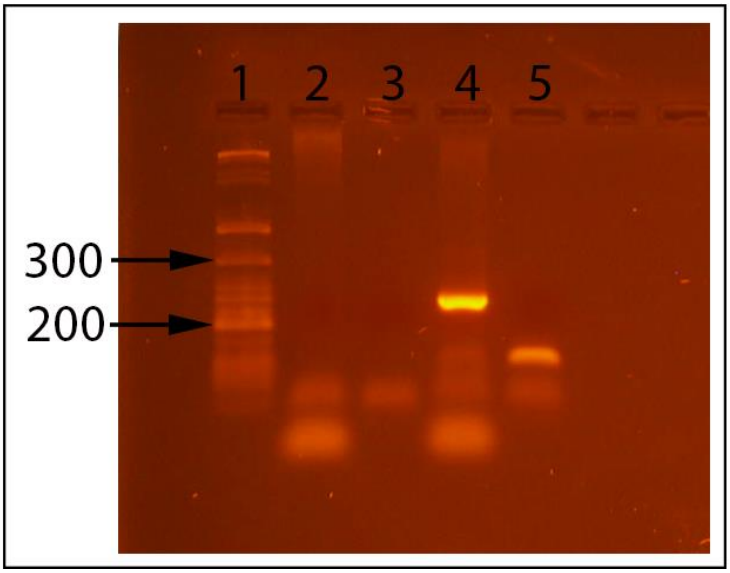


**Figure 2.4.4.1: PCR products visualised on agarose gel. 1 = DNA ladder, 2 = Primer set 1, 3 = Primer set 1 no template control, 4 = Primer set 2, 5 = Primer set 2 no template control. Arrows show base pair size on ladder. Primer Set 2 has resulted in a clear product of the expected 247 base pair size**.

### 2.4.4 DNA sequencing

Samples were sent to Genetic Analysis Services at the University of Otago. It was confirmed that the primers produced were of the correct nucleotide sequence.

### 2.4.5 Probe synthesis

The digoxigenin (DIG) labelled probes were made using the DIG RNA labelling and detection kit (Roche Applied Science, Penzberg, Germany). Two sets of PCR products were used, the first from Primer 2 identified in the table (Probe A), and the second probe (Probe B) was designed by Dr Hollian Phillips (Department of Anatomy, University of Otago). Each PCR product was used to make an antisense and sense (control) probe.

A mass of 200 ng of the PCR template was used for each probe (2.9 µl for Probe A, 2.2 µl for Probe B) along with 2 µl DIG RNA labelling and 2 µl 10 X transcription buffer. The antisense probes used 2 µl of RNA Polymerase T7 and the sense probes used 2 µl of RNA Polymerase SP6. All tubes were made up to 20 µl by using nuclease free water. After pipetting to mix, they were briefly centrifuged and incubated in 37°C heat block for 2 hours. Following incubation, 1 µl of DNase was added, followed by a further 15 minutes at 37°C. To stop the reaction, 2 µl of EDTA was used, and then 27 µl TE buffer was used to make it up to 50 µl. For purification, it was run through a Mini Quick Spin RNA column (Roche Applied Science, Penzberg, Germany). The probes were stored at -80°C until use.

Following DIG labelling, the concentrations of the probes were checked using a dot blot. This process estimated the concentrations of the probes to be between 15-20 ng/µl. These numbers were used in calculating the probe-hybridisation buffer mixture.

### 2.4.6 In situ hybridisation protocol

*2.4.7.1 Tissue preparation*

Slides were taken out of the -20°C freezer and carried on dry ice to a 55°C oven, where they thawed for 5 minutes. Post fixation in 2% paraformaldehyde for 5 minutes at RT was performed. Slides were washed in 0.5 x SSC for 5 minutes and then immersed in 2 µg/ml proteinase K solution for 10 minutes in order to digest the tissue sufficiently to allow for probe hybridisation. Three washes followed: 0.5 x SSC for 10 minutes, 1 x TEA for 3 minutes and 1 x TEA with acetic anhydride. The acetylation occurs on the positively charged amino acids to prevent background binding with the negatively charged probes. Slides were washed twice in 2 x SSC for 2 minutes. Sections were dehydrated by immersion in 50% ethanol for 1 minute, 80% ethanol for 1 minute, 95% ethanol for 2 minutes, 100%

ethanol for 1 minute, chloroform for 5 minutes (to remove lipids), 100% ethanol for 1 minute and 95% ethanol for 1 minute. The slides were allowed to air dry for at least 1 hour.

*2.4.7.2 Hybridisation*

Hybridisation buffer was thawed and DTT was added to give a final concentration of 100mM. For each slide, 0.8 µl of probe and 1 µl of tRNA was needed. Each probe and tRNA mixture was heated at 95°C on the heat block for 3 minutes for denaturing, and then immediately put into ice cold hybridisation buffer (enough to make up to volume 120 µl for each slide) and pipetted to mix. The 120 µl was put on each slide and covered with a hybridisation cover slip. They were placed elevated in hybridisation boxes lined with paper towels soaked in box buffer to maintain humidity. The slides were incubated at 55°C for 18 hours.

*2.4.7.3 Washing*

The slides were taken out of the oven and left in the fumehood for 5 minutes. They were soaked in 2 x SSC until the coverslips came off, and were washed twice in 2 x SSC for 10 minutes. Non-specific background staining was reduced by immersion in 2% RNase A solution for 30 minutes. Slides were washed twice in 2 x SSC for 10 minutes, then washed in 0.1 x SSC in a 65°C water bath for 2 hours (solution changed halfway through). After the hot wash to reduce non-specific hybridisation, slides were put in 0.5 x SSC for 10 minutes, and twice in Buffer 1 for 5 minutes.

*2.4.7.4 Antibody labelling*

Sections were blocked with DIG blocking solution diluted 1:5 in Buffer 1 with 1% triton for 30 minutes. They were incubated with DIG-AP antibody diluted 1:2000 in blocking solution at 4°C for 36-48 hours.

Sections were washed twice in Buffer 1 for 10 minutes, and once in Buffer 3 for 10 minutes.

*2.4.7.5 Staining*

NBT/BCIP solution was applied onto slides and they were left to incubate at RT. Colour development time varied as specified in results.

Slides were washed 4 times in 1 x SSPE for 30 minutes. They were rinsed in distilled $H_2O$, then went through ethanol dilutions: 70%, 95% and 100% for 1 minute each. They were dried on a slide warmer for 1 hour at 42°C, and then coverslipped with Vectamount permanent mounting medium (Vector Laboratories, California, USA).

## 2.5 Aromatase radioactive in situ hybridisation

### 2.5.1 Probe synthesis

The sulphur-35 (S-35) labelled probes were made using the Riboprobe in vitro transcription kit (Promega, Wisconsin, USA). PCR products used for these probes were made from primers designed by Dr Hollian Phillips (Department of Anatomy, University of Otago) using protocol as per non-radioactive probes. This was the same primer set that was designated Probe B for non-radioactive in situ hybridisation. PCR product was visualised on agarose gel (Figure 2.3.2.1).

Two µl each of ATP, GTP, CTP, RNase-free water, and 8 µl of S-35 labelled UTP were put in a speed vacuum on medium heat for 16 minutes. Added to this and vortexed to mix were 2 µl each of 5 x transcription buffer, nucleotide mix, PCR product, RNase-free water, and 1 µl of 100mM DTT. For the antisense probe 1 µl of T7 RNA polymerase was added, while for the sense probe 1 µl of SP6 RNA polymerase was added. For both probes, 0.5 µl of Rnasin ribonuclease inhibitor was added, followed by incubation at 37°C for 2 hours. After this incubation, 0.2 µl of DNase was added, followed by further incubation at 37°C for 15 minutes. The reaction was stopped by 1 µl of 0.25M EDTA, and 38 µl of TEA buffer was added to make the probe volume up to 50 µl.

To purify the probe, it was put through a Mini Quick Spin RNA column twice (Roche Applied Sciences, Penzberg, Germany). Volume was made up to 100 µl by TEA buffer, and the probe was kept at -80°C for a maximum time of 1 week. The incorporation of S-35 with the probe was measured by a scintillation counter. An incorporation of 60% was deemed ideal.

### 2.5.2 Radioactive in situ hybridisation protocol

*2.5.2.1 Tissue preparation*

Slides were taken out of the -20°C freezer and carried on dry ice to a 55°C oven, where they thawed for 5 minutes. Post fixation in 2% paraformaldehyde for 5 minutes at RT was performed. Slides were washed in 0.5 x SSC for 5 minutes and then immersed in 2 µg/ml proteinase K solution for 10 minutes in order to digest the tissue sufficiently to allow for probe hybridisation. Three washes followed: 0.5 x SSC for 10 minutes, 1 x TEA for 3 minutes and 1 x TEA with acetic anhydride. The acetylation occurs on the positively charged amino acids to prevent background binding with the negatively charged probes. Slides were washed twice in 2 x SSC for 2 minutes. Sections were dehydrated by immersion

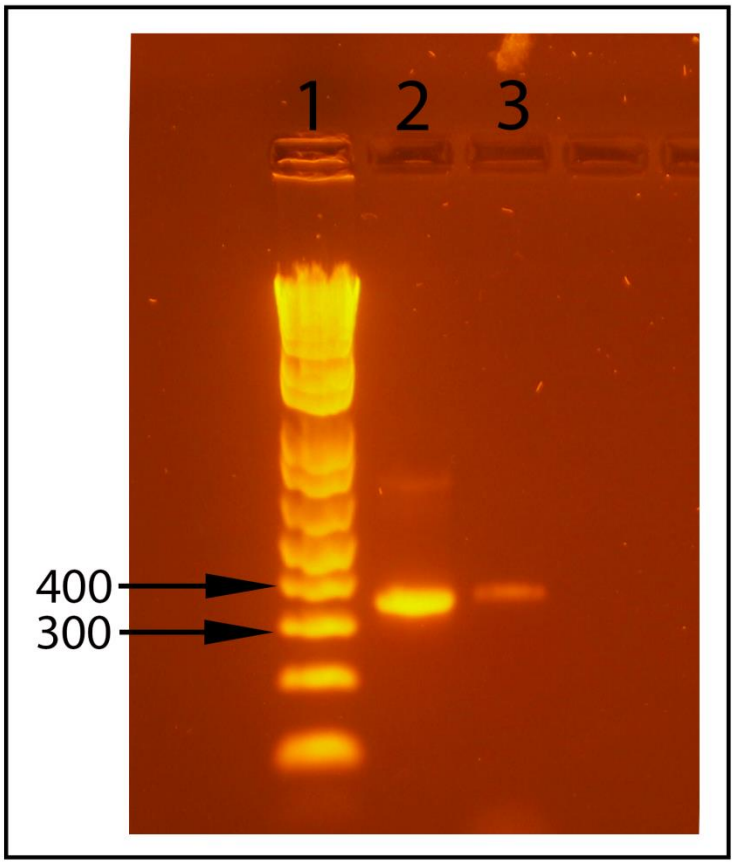


**Figure 2.5.1.1: PCR product visualised on agarose gel. 1 = DNA ladder, 2 = Probe B primers, 3= no template control. Arrows show base pair size on ladder. Product is of the expected size of 344 base pairs.**

in 50% ethanol for 1 minute, 80% ethanol for 1 minute, 95% ethanol for 2 minutes, 100% ethanol for 1 minute, chloroform for 5 minutes (to remove lipids), 100% ethanol for 1 minute and 95% ethanol for 1 minute. The slides were allowed to air dry for at least 1 hour.

*2.5.2.2 Hybridisation*

The probe was removed from the freezer and centrifuged at 4°C at 12000G for 20 minutes. Supernatant was poured off, and 500 µl ice cold ethanol was added. This was centrifuged again at 4°C at 12000G for 5 minutes. Supernatant was pipetted off, and the probe was left to air dry for 20 minutes. The pellet was dissolved in TE buffer, and samples taken to be read by the scintillation counter to ensure they were above 60,000 counts.

For each slide, 2 µl of probe and 1 µl of tRNA was needed. Each probe and tRNA mixture was heated at 95°C on the heat block for 3 minutes for denaturing, and then immediately put into ice cold hybridisation buffer (enough to make up to volume 120 µl for each slide) and pipetted to mix. The 120 µl was put on each slide and covered with a hybridisation cover slip. They were placed elevated in hybridisation boxes lined with paper towels soaked in box buffer to maintain humidity. The slides were incubated at 55°C for 18 hours.

*2.5.2.3 Washing*

The slides were taken out of the oven and left in the fumehood for 5 minutes. They were soaked in 2 x SSC until the coverslips came off, and then were washed twice in 2 x SSC for 10 minutes. They were then immersed in 2% RNase A solution for 30 minutes to reduce non-specific background staining. Slides were washed twice in 2 x SSC for 10 minutes, then washed in 0.1 x SSC in a 65°C water bath for 2 hours (solution changed halfway through). Slides were washed twice in 0.5 x SSC for 10 minutes each. They were then put through ethanol washes: 50%, 80%, 95% and 100% for 3 minutes each and dried overnight.

*2.5.2.4 Film development*

Slides were put into cassettes with film for 2 weeks at 4°C. After film development, the slides were kept at RT until emulsion.

*2.5.2.5 Emulsion*

Slides were dipped in radiographic emulsion (Ilford Nuclear Emulsion K5, Agar Scientific, Stansted, Essex) at 40°C, air dried vertically for 15 minutes and horizontally for 1 hour at RT. They were placed into slide boxes which were put into light-proof metal tins containing desiccant. The slides were stored at 4°C for at least 4 weeks.

Slides were immersed in slide developer solution (Kodak D-19 Replacement, EMS, Pennsylvania, USA) at 20°C for 8 minutes, agitated every minute. They were then put into tap water at 20°C for 30 seconds as a stop solution. This was followed by 10 minutes with gentle agitation in a fix solution (30% sodium thiosulfate), and finally slides were washed for 20 minutes in running tap water.

Counterstaining was performed using cresyl violet. Slides were put through 10 seconds of 70% ethanol, 95% ethanol, 70% ethanol, $H_2O$ and then 30-40 seconds in 0.1% cresyl violet solution. Slides were destained for 20 seconds in 100% ethanol.

Slides were placed on a slide warmer at 42°C for 1 hour, put into xylene for 5 minutes twice and coverslipped using Vectamount permanent mounting medium (Vector Laboratories, California, USA).

## 2.6 Smad 1/5/8 Immunohistochemistry

Immunohistochemistry is a technique based on the principle that specific antibodies bind to their specific antigen. This specific antigen for this procedure is phosphorylated Smad 1/5/8, an intracellular signalling molecule of AMH.

### 2.6.1 Immunohistochemistry Protocol

#### *2.6.1.1 Tissue Preparation*

Brain sections were cut at 16µm at -25°C and thaw mounted onto Superfrost Plus slides (LabServ, Thermo Fisher Scientific, Massachusetts, USA). They were kept at -20°C until use, when they were thawed for 5 minutes at room temperature. They were washed twice in glycine in phosphate buffer for 5 minutes each, and then twice in 0.01M phosphate buffer (PB) for 5 minutes each. Sections were incubated with 5% Donkey serum (Sigma-Aldrich, Missouri, USA) diluted in primary antibody diluting buffer for 20 minutes at RT.

#### *2.6.1.2 Antibody labelling*

Phosphorylated Smad 1/5/8 antibody (Chemicon, Merck-Millipore, Massachusetts, USA) diluted at 1:100 in primary antibody diluting buffer was added to the sections, and incubated at 4°C overnight. Control sections were incubated with Rabbit IgG antibody (Vector Laboratories, California, USA) at 1:1000 dilution.

Sections were washed 6 times in wash buffer for 5 minutes each and incubated with Donkey Anti-Rabbit biotinylated antibody (Jackson Immunologicals, Pennsylvania, USA) diluted at 1:500 (concentration unknown) for 1.5 hours. They were washed 3 times in wash buffer for 5 minutes. A 1% hydrogen peroxide solution was applied for 10 minutes, and washed in 0.01M PB for 5 minutes.

#### *2.6.1.3 Staining*

Streptavidin-Biotinylated Horseradish Peroxidase complex (Amersham, GE Healthcare, Buckinghamshire, UK) diluted 1:100 with secondary antibody diluting buffer was applied for 1 hour. Sections were washed 3 times in 0.01M PB for 1 minute each. 3,3'-Diaminobenzidine (DAB) solution (DAB substrate kit, Vector Laboratories, California, USA) was applied for 12 minutes and then sections were washed in tap water and distilled water for 5 minutes each. Glue was removed and slides were left to dry before being dipped in xylene and coverslipped with Entellan (Merck-Millipore, Massachusetts, USA).

# 3. Results

## 3.1 Aromatase non-radioactive in situ hybridisation

Initial non-radioactive in situ hybridisation was performed using a selection of brain tissue, with ovary and testes used as positive controls. The time needed for colour development was unknown, so the sections were checked every two hours up to 6 hours, then every two hours the following day. There was no clear positive staining initially and background staining could be seen at 2 hours. Most DIG labelled protocols leave colour development for 2-4 hours, so prominent background staining at 2 hours was unexpected.

One explanation for the high background staining was non-specific antibody binding causing increased detection from the NBT/BCIP colour development procedure. To investigate this, the in situ hybridisation protocol was repeated, from *Tissue preparation (2.4.7.1)* through to *Staining (2.4.7.5)* but omitting the *Hybridisation (2.4.7.2)* steps. However, slides with no antibody, anti-DIG antibody and increased antibody concentration all had equal levels of background staining, concentrated around the section edges and striatum. Five different colour development times were trialled: 1 hour, 2 hours, 4 hours, 6 hours and overnight. This troubleshooting procedure showed that substantial background staining was present at 1 hour which was independent of antibody concentration. This suggests that it is not a problem of the anti-DIG antibody binding non-specifically.

Several blocking procedures were trialled against endogenous phosphatases in order to reduce the background staining. Hydrogen chloride (HCl) and levamisole ((6S)-2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-b]thiazole, monohydrochloride) were used, followed by NBT/BCIP colour development for either 10 minutes or 1 hour. The maximum time for development was based on the high background seen in the last experiment. Both HCl and levamisole were effective in blocking endogenous phosphatases compared to control at both time points, but HCl was more effective at 1 hour (Figure 3.1.1). The use of either solutions could therefore be used to improve signal-to-noise ratio.

The effectiveness of the probes could now be investigated. Both Probe A and Probe B were trialled, along with no probe controls. Levamisole was included in all NBT/BCIP solutions, and a subset of sections had an additional HCl step applied. Some sections had their colour development stopped at 1 hour (Figure 3.1.2A) where light background staining could be observed. The remainder of the sections were stopped at 3.5 hours, at which time moderate background staining could be observed in many sections.

No clear positive staining was observed in any of the sections. There was no major colour difference between the antisense and sense probes, while no probe controls tended to have less staining. Background staining was reduced by HCl application. The difference in the level of staining at 1 hour between Figure 3.1.1E and Figure 3.1.2A is likely due to the full in situ hybridisation protocol that the sections in Figure 3.1.2 underwent.

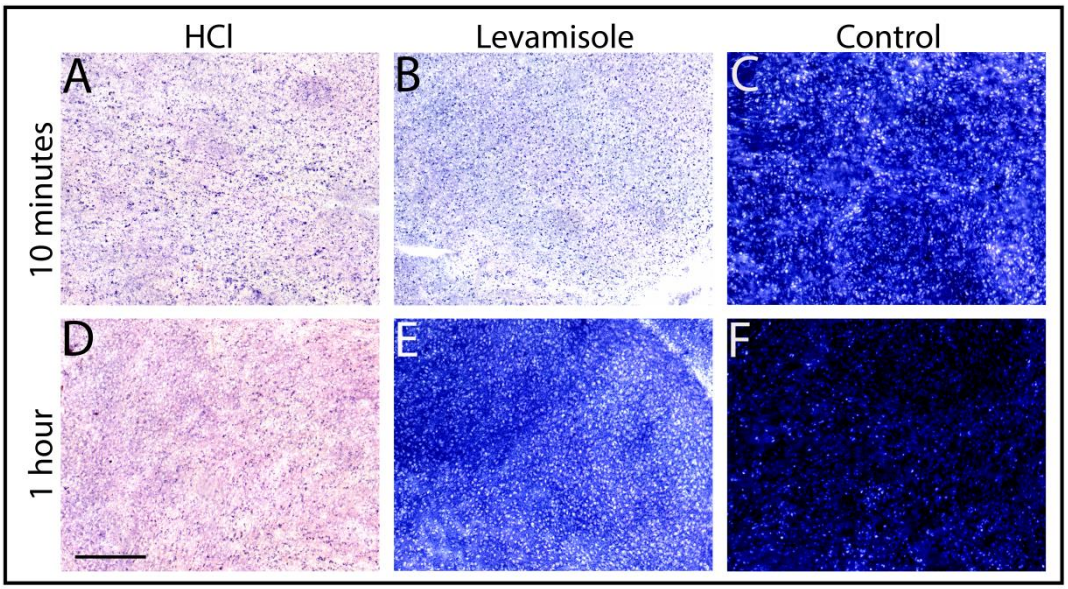


**Figure 3.1.1: Comparison of endogenous phosphatase blocking solutions. At 10 minutes, both HCl (A) and levamisole (B) have greatly reduced background compared to control (C). Using HCl, the background staining does not increase greatly from 10 minutes (A) to 1 hour). However, at 1 hour, the use of levamisole still decreased background staining compared to control (F). The background staining for levamisole at 1 hour is similar to control at 10 minutes. Images were captured at 100X magnification, scale bar = 200µm**

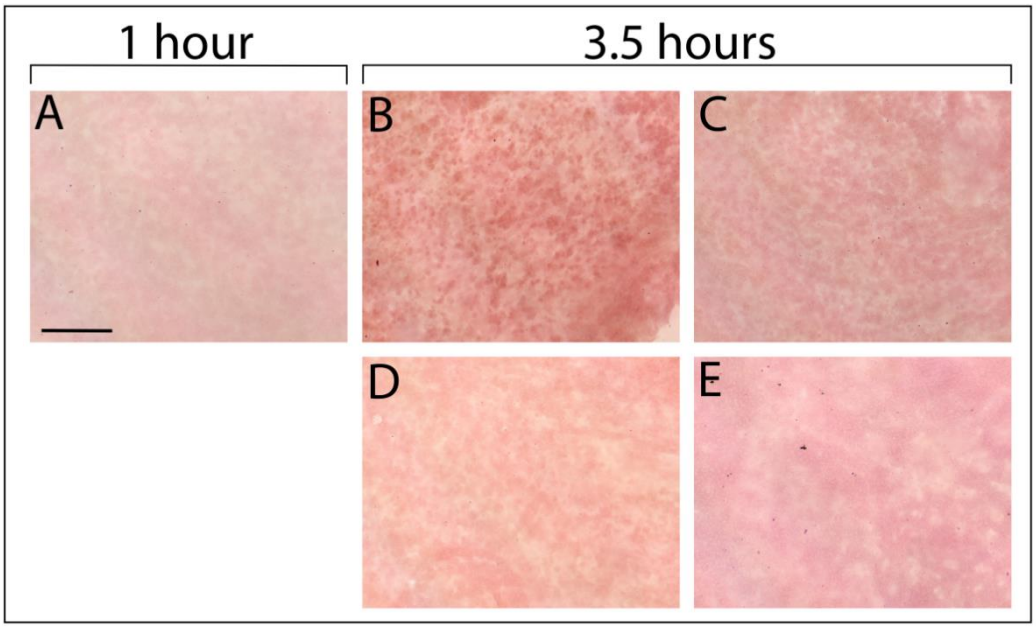


**Figure 3.1.2: Tissue sections developed at 1 hour and 3.5 hours using Probe B. A and B = antisense probe, C = sense probe, D = antisense with HCl, E = no probe control. There is an increase in background staining in the antisense sections from 1 hour to 3.5 hours, which is delayed by the application of HCl. The no probe control at 3.5 hours has a similar amount of background to the antisense at 1 hour. The antisense and sense at 3.5 hours have similar levels of background staining. Images were captured at 100X magnification, scale bar = 200µm.**

## 3.2 Aromatase radioactive in situ hybridisation

Radioactive in situ hybridisation revealed positive aromatase staining in all experimental groups. Staining in the BNST occurred in the medial division, posteromedial part and posterointermediate part (Figure 3.2.1). There was wide variation of staining intensity within each genotype (Figure 3.2.1). At all age groups, the AMHR2$^{-/-}$ male mouse had staining intensity that was very similar with male AMHR2$^{+/+}$ mice. This indicated that a level of aromatase expression is present in male AMHR2$^{-/-}$ mice.

Radioactive in situ hybridisation was only successful for one 2 day old AMHR2$^{+/+}$ female (Figure 3.2.1G) so valid comparisons with this group cannot be made. Staining intensity observed in this female was lower than the two male groups. This is consistent with previous reporting of aromatase expression (64). One AMHR2$^{+/+}$ female was sectioned incorrectly so only the rostral part of the BNST was included, which had no staining (results not shown).

The rostral to caudal staining pattern in the BNST occurred in a consistent pattern in the experimental animals (Figure 3.2.2). There was no staining in the rostral part, positive signal occurred approximately midway through the BNST in the rostral-caudal direction. This continued up to near the caudal end. For each section, the staining intensity was highest in the middle of the distribution.

The 20 day old and 42 day old groups underwent greater than one half-life of sulfur-35 (87 days) before emulsion coating could be performed. This was due to a delay in the supply of the radiographic emulsion solution. The slides developed therefore did not have adequate signal for comparisons to be made (Figure 3.2.4). Radiographic film images were of poor resolution and positive signal was difficult to identify, with significant background in some sections (Figure 3.2.3). Positive signals seen on film were not replicated following emulsion exposure.

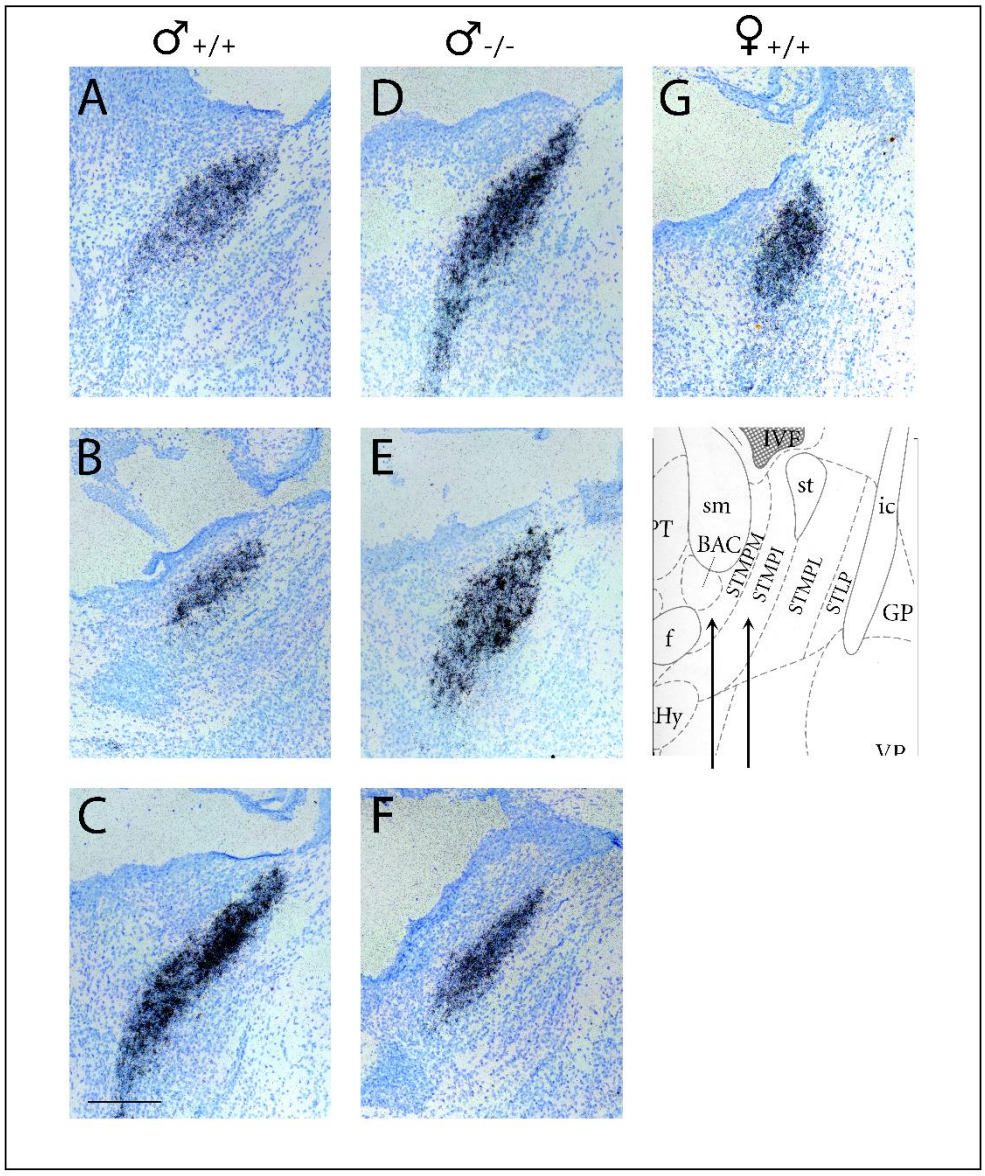


**Figure 3.2.1: Aromatase radioactive in situ hybridisation performed on 2 day old mice. Sections shown are at the same coronal level containing BNST. A, B and C are AMHR2$^{+/+}$ males. D, E and F are AMHR2$^{-/-}$ males and G is an AMHR2$^{+/+}$ female. The arrows show the area of the BNST where staining is present, STMPM = BNST medial division, posteromedial part. STMPI = BNST medial division, posterointermediate part. Diagram has been adapted from Paxinos (96). There is aromatase staining in all genotypes with variation within genotypes. C has more intense staining than A and B though they are all AMHR2$^{+/+}$ males. There is also considerable overlap in staining between genotypes, D has similar staining intensity to C. Images were captured at 100X magnification, scale bar = 200µm.**

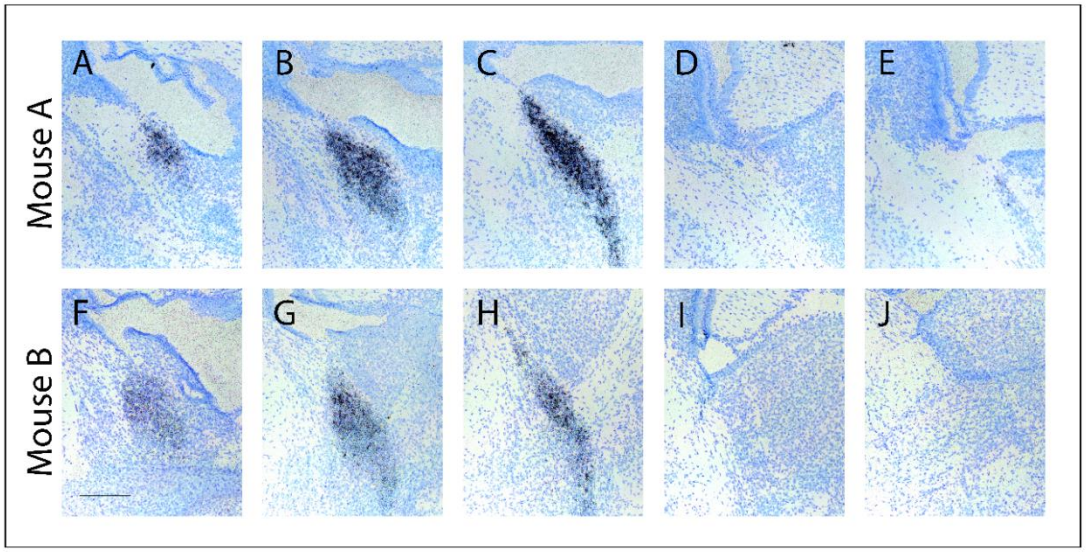


**Figure 3.2.2: Aromatase radioactive in situ hybridisation performed on 2 day old mice. A to E are serial sections from an AMHR2$^{-/-}$male mouse. F to J are serial sections from an AMHR2$^{+/+}$ male mouse. Sections are separated by 32µm and are arranged in rostral to caudal orientation from left to right. Positive staining occurs approximately halfway through the BNST and continues until near the caudal end. This was the pattern seen in positive staining throughout the BNST and was consistent throughout the genotypes, albeit with differences in staining intensity. Images were captured at 100X magnification, scale bar = 200µm.**

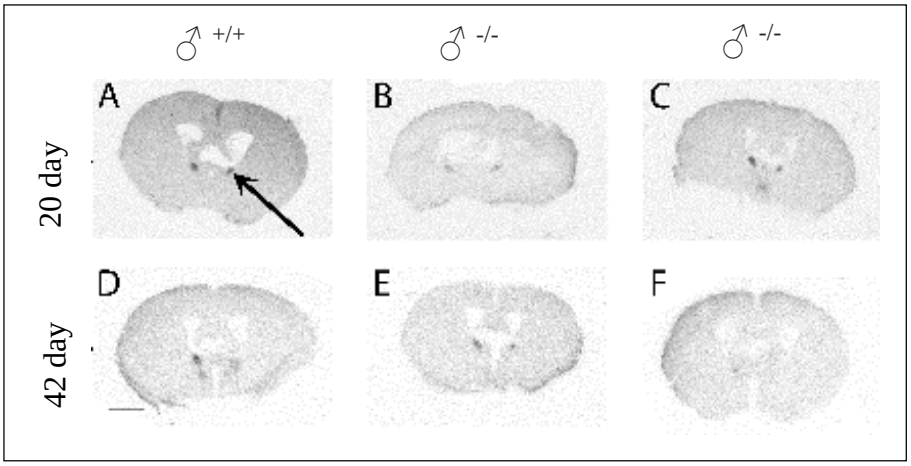


**Figure 3.2.3: Radiographic films of radioactive in situ hybridisation performed on 20 (A, B, C) and 42 day old mice (D, E, F). Arrow in A shows the positive staining in the region of the BNST. Radiographic films were scanned at full size, scale bar = 2mm**

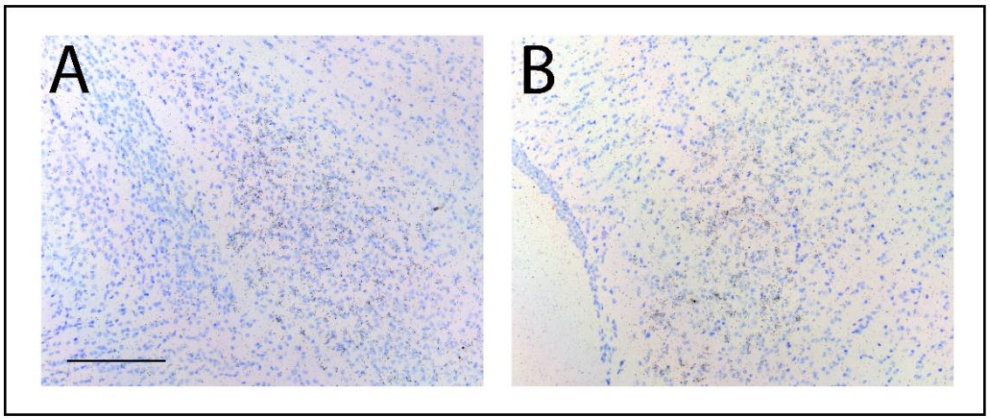


**Figure 3.2.4: Radioactive in situ hybridisation performed on AMHR$^{+/+}$ mice. A is 20 days old and B is 42 days old. Very light positive staining can be seen. Images were captured at 100X magnification, scale bar = 200µm**

## 3.3 Smad 1/5/8/ Immunohistochemistry

Immunohistochemistry for phosphorylated Smad 1/5/8 molecules was commenced using practice tissue from male AMHR2$^{+/-}$ mice. Positive staining was obtained in the region of the BNST, and the staining appeared to be located to the nucleus. This is consistent with the cellular location of phosphorylated Smad 1/5/8.

The immunohistochemistry was unable to be continued due to the manufacturer's discontinuation of the antibody.

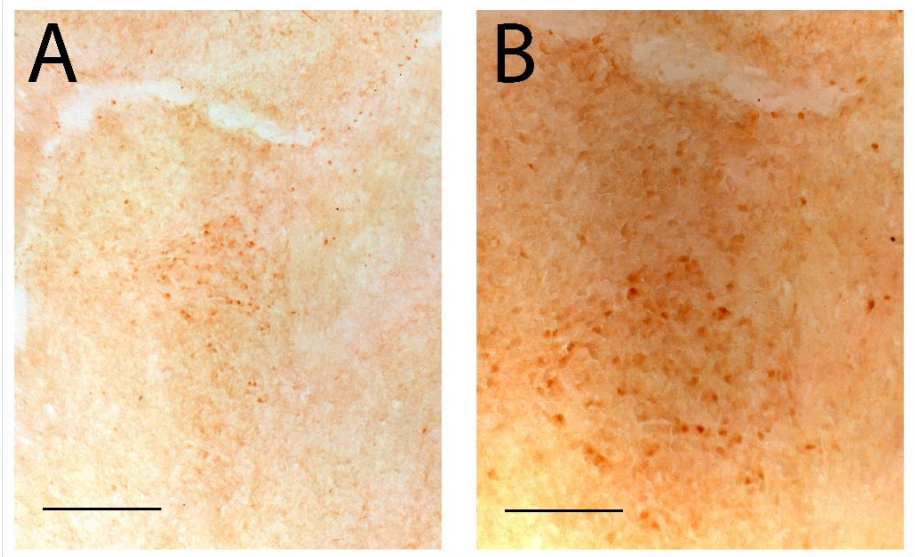


**Figure 3.3.1: Phosphorylated Smad 1/5/8 immunohistochemistry performed on a male AMHR2$^{+/-}$ mouse. Nuclear staining can be seen in the region of the BNST. A is taken at 100X magnification, scale bar = 200µm. B is taken at 200X magnification, scale bar = 100µm.**

# 4. Discussion

## 4.1 Aromatase Expression

The results obtained from the study show that AMHR2$^{-/-}$ mice express aromatase at a level similar to AMHR2$^{+/+}$ mice at 2 days old. Within each genotype there was wide variation in the aromatase expression in the same area of the BNST. This suggests that AMH is working through a pathway independent of aromatase to alter brain anatomy because aromatase expression appears to be unaffected by the lack of AMH.

However, we must consider whether or not the similar aromatase expression level between the AMHR2$^{+/+}$ and AMHR2$^{-/-}$ male mice is a true result. There were only 3 animals for each genotype, insufficient to perform statistics. Expression at 20 days old (pre-pubertal) and 42 days old (adult) was unable to be examined properly due to insufficient staining. The mice with high aromatase expression could have been outliers, or they could lie in the normal range. Alternately, the experimental technique could be variable: the probe is not hybridising effectively on every section, radiographic emulsion was not applied correctly or positive signal was washed away during slide development. Increasing the number of animals in each genotype group would give us a more representative sample of the animals, and allow us to see if the variability in aromatase expression is a true biological effect.

There is also the consideration of how much of an effect on brain structure a reduced aromatase level would cause. Testosterone levels peak at birth (60) but this has not been measured in the AMHR2$^{-/-}$ mouse. Nonetheless, 5 day old AMH$^{-/-}$ mice were found to have testosterone levels in the normal range (7). This means that the substrate for the aromatase enzyme is present. An adequate amount of oestradiol may still be produced to exert an effect on brain anatomy. AMH may need to have a major effect on aromatase for changes to occur in the AMHR2$^{-/-}$ male. The AMHR2$^{-/-}$ males have similar BNST neuron numbers to a female prior to puberty (8), but female mice have very low levels of testosterone. It is still possible aromatase expression is different in the AMHR2$^{-/-}$ mice; this would also be confirmed by increasing the sample size.

## 4.2 Future Investigation of Aromatase

Beyond increasing sample size, there are other investigations that could be performed to validate the variability of aromatase expression seen in the present study. Immunohistochemistry with the aromatase enzyme was also attempted (results not shown) but remained unsuccessful after modifications of the protocol. Immunohistochemistry results alongside in situ hybridisation would have been extremely useful because the presence of mRNA does not always directly correlate to a final translated protein (97, 98). There are 'slow' codons in mRNA that correspond to rare tRNAs or amino acids that can inhibit protein production, slowing the rate of translation by six fold (99, 100). The aromatase amino acid sequence was found to have 177 'slow' amino acids out of 503. Although this may not be directly applicable to murine protein translation, it presents a possible situation where the aromatase mRNA level may be higher than the protein product. This would mean that in situ hybridisation may not be reflective of what is truly happening in the tissue, thus immunohistochemistry would be valuable to corroborate results.

Post-transcriptional regulation of the aromatase mRNA could be different in the $AMHR2^{-/-}$ mice. Similar levels of aromatase mRNA in $AMHR2^{-/-}$ and wild type mice may lead to different amounts of aromatase enzyme. There are many processes involved in post-transcriptional modification and there are also micro RNA molecules (miRNAs) that repress gene expression. Identification of their individual function is difficult as they can regulate hundreds of genes each (101). But research into miRNAs is being expanded widely, and it can be identified using in situ hybridisation (102). In the future it may be that the miRNA control of aromatase could be investigated in the $AMHR^{-/-}$ mice. Any possible post-transcriptional regulation that AMH has on aromatase could then be identified.

## 4.3 AMH Pathways

The effect of AMH on neurons could be investigated to explore the mechanisms of the aromatase-independent pathway. One of the results observed in this pathway is the male-typical increase of neuron size and number in the BNST (8). Therefore, it will be of great interest to see the distribution of AMH intracellular signalling molecules (phosphorylated Smad 1/5/8) in the BNST using immunohistochemistry. Positive staining was observed in the BNST in $AMHR2^{+/-}$ mice but was stopped as the manufacturers discontinued the antibody. The next step would be to find and optimise another suitable antibody.

Immunohistochemistry would need to be performed on both $AMHR2^{+/+}$ and $AMHR2^{-/-}$ mice because Smad 1/5/8 are not specific to AMH alone. They are phosphorylated by other members of the TGF-β superfamily (52). A reduced level of staining in the $AMHR2^{-/-}$ mice would suggest that the phosphorylated Smad 1/5/8 in the $AMHR2^{+/+}$ is largely due to AMH. The neurons responding to AMH could then be identified. From here, we could look at the consequences that AMH has on the cell. Analysis of the RNA from these cells responding to AMH could be compared to cells not exposed to AMH. DNA microarrays could be performed, serial analysis of gene expression (SAGE) could be used or next generation sequencing techniques could be applied for the mRNA transcriptome. These techniques would detect which genes are being transcribed following AMH receptor activation. Exploring what function these genes serve would help us uncover what mechanism AMH is acting through to cause its effect on the brain.

## 4.4 Non-radioactive in situ hybridisation methodology

### 4.4.1 DIG labelled in situ hybridisation

Radioactive in situ hybridisation provides high sensitivity in detection of low abundance mRNA, and was successful in this study. However, it comes with biohazard risks, long radiographic exposure times, probe instability and considerable expense (103-105). Non-radioactive in situ hybridisation is a useful alternative. It costs less, has no risk of radiation exposure and has a much faster turnaround. In addition, it provides a higher degree of cellular resolution (103, 104). This would allow the precise cellular localisation of the aromatase mRNA. However, after several troubleshooting procedures a clearly positive signal was not able to be achieved using DIG labelled in situ hybridisation.

In situ hybridisation is an extensive and complex technique. There are multiple factors that could have led to the failure of the DIG-labelled version of the technique. The visualisation process of the anti-DIG antibody followed by the NBT/BCIP colour reaction appeared to be too insensitive to confirm clearly positive hybridisation. Doubling concentrations or omitting the anti-DIG antibody caused no changes to the level of background staining. This suggests that the background was not due to the antibody. The application of anti-DIG antibody was also trialled on probe-blank sections, demonstrating that non-specific binding of the antibody to endogenous antigens was not occurring. Previous studies have shown that non-specific DIG binding is low in mammalian cells (106). Given more time, different anti-DIG antibody concentrations could have been trialled to improve its chances of binding to the DIG-labelled probe.

### 4.4.2 Probe hybridisation

Probe B was shown to hybridise specifically with aromatase mRNA in radioactive in situ hybridisation, but Probe A was not used to conserve time and resources. It remains a possibility that Probe A is hybridising non-specifically with incorrect mRNA sequences, or not hybridising at all. Probe hybridisation can depend on its length, the temperature and the composition of the hybridisation buffer. A longer probe has been shown to result in more stable hybrids with the mRNA (107) while shorter probes diffuse more easily through the tissue (108) and can also use a shorter hybridisation time (109, 110). A different probe, binding to a different nucleotide sequence on the aromatase mRNA could be designed; perhaps more relaxed criteria could be used in the selection of the sequence to give more options.

The nucleotide sequence of Probe A may have not been ideal for the aromatase mRNA, and the hybridisation temperature may not have been optimal. The melt temperature ($T_m$ - the temperature at which 50% of all possible hybrids are formed, and 50% still remain single stranded) given for Probe B on Primer-BLAST was 60°C. The hybridisation temperature used was 55°C, consistent with being at least 5°C lower than the $T_m$ (109). Lower temperatures are widely used so it could be advantageous to trial lower hybridisation temperatures. The $T_m$ is also affected by sodium chloride (NaCl) concentration (108). Optimisation of the NaCl concentration in the hybridisation buffer will assist in stabilising the hybrids formed. A doubling of the NaCl concentration was trialled in the present project but no improvement was observed (results not shown). This could be repeated with different concentrations and properly controlled to determine the optimal NaCl concentration.

For the probe to successfully hybridise with the mRNA of interest, it needs to be able to diffuse through the section, and the mRNA must be unrestricted by nearby proteins. The current protocol includes a chloroform step in the tissue preparation phase to remove lipids, and a Proteinase K digestion step. Many published protocols advocate adjusting the temperature and duration of the Proteinase K step according to the fixation method and tissue type (111-113). Given the range of different protease digestion protocols that exist for in situ hybridisation, it would be rational to try a range of concentrations for different amounts of time, and at both RT and 37°C. A post-fixation step following the protein digestion has also been shown to be useful in preserving the mRNA present in the section (108).

### 4.4.3 Signal amplification

There are many options for amplification of the labelling observed, some more effective than others. Amplification of the target mRNA sequence (in situ PCR) is one that is theoretically simple. It seems ideal particularly for aromatase mRNA which has been shown to have a short half-life of 3 hours in bovine granulosa cells (114). In situ PCR would counteract the early loss of nucleic acids prior to hybridisation. These methods apply PCR techniques prior to in situ hybridisation. But in practice, in situ PCR often results in low amplification efficiency, poor reproducibility and difficulty in quantification of results (115, 116). A similar technique which incorporates labelled nucleotides into the PCR reaction (eliminating the need for in situ hybridisation) has problems with incorporation of labelled nucleotides into non-specific PCR products, including endogenous DNA undergoing repair

(116). These in situ PCR methods are not widely used due to these problems, and it would be considered a last resort for this study.

Amplification of the signal post-hybridisation is a more widespread method of increasing detection. A popular version of this is tyramide amplification. This is performed following DIG in situ hybridisation, with a conjugated anti-DIG horseradish peroxidase (HRP) antibody. Activated biotinylated tyramine is then deposited at or near the HRP site and its biotin sites act as further binding sites for streptavidin-biotin complexes which are then visualised (115, 116). This can result in an amplification factor of 5-10 fold with accurate localisation of signal (117). However, when signal amplification is performed, background is also increased so it should be used with a probe that is known to be hybridising specifically. This could be applied to a DIG in situ hybridisation procedure using Probe B.

## 4.5 Summary

Overall, there was wide variation of aromatase expression within genotypes, and overlap in expression between male AMHR2$^{+/+}$ and AMHR2$^{-/-}$ mice. This supports the concept of AMH acting through an aromatase-independent pathway to exert changes in brain anatomy. However, sample size was very small and the in situ hybridisation procedure was fully completed on the 2 day old mice only. Further investigations to confirm this include immunohistochemistry for aromatase and phosphorylated Smad 1/5/8, and genomic RNA studies.

Radioactive in situ was the only technique that was successful within the time frame. Probe design, hybridisation temperatures, hybridisation buffer, protease digestion, and signal amplification could all be explored given an opportunity to further optimise the DIG in situ hybridisation. Some of these techniques like protease digestion and hybridisation buffer could also be applicable to radioactive in situ hybridisation to achieve a stronger signal more quickly.

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