

# Anti-Müllerian hormone is a gonadal cytokine with two circulating forms and cryptic actions

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## Abstract

Anti-Müllerian hormone (AMH) is a multi-faceted gonadal cytokine. It is present in all vertebrates with its original function in phylogeny being as a regulator of germ cells in both sexes, and as a prime inducer of the male phenotype. Its ancient functions appear to be broadly conserved in mammals, but with this being obscured by its overt role in triggering the regression of the Müllerian ducts in male embryos. Sertoli and ovarian follicular cells primarily release AMH as a prohormone (proAMH), which forms a stable complex (AMH<sub>N,C</sub>) after cleavage by subtilisin/kexin-type proprotein convertases or serine proteinases. Circulating AMH is a mixture of proAMH and AMH<sub>N,C</sub>, suggesting that proAMH is activated within the gonads and putatively by its endocrine target-cells. The gonadal expression of the cleavage enzymes is subject to complex regulation, and the preliminary data suggest that this influences the relative proportions of proAMH and AMH<sub>N,C</sub> in the circulation. AMH shares an intracellular pathway with the bone morphogenetic protein (BMP) and growth differentiation factor (GDF) ligands. AMH is male specific during the initial stage of development, and theoretically should produce male biases throughout the body by adding a male-specific amplification of BMP/GDF signalling. Consistent with this, some of the male biases in neuron number and the non-sexual behaviours of mice are dependent on AMH. After puberty, circulating levels of AMH are similar in men and women. Putatively, the function of AMH in adulthood maybe to add a gonadal influence to BMP/GDF-regulated homeostasis.

## Key Words

- ▶ Sertoli cells
- ▶ whole animal physiology
- ▶ development
- ▶ female reproduction
- ▶ male reproduction

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## Introduction

Anti-Müllerian hormone (AMH, Müllerian inhibiting substance) is part of the classical pathway for the induction of the male phenotype. It is the testicular secretion that triggers the degeneration of the uterine precursor (Müllerian duct) in male embryos (MacLaughlin & Donahoe 2004, Josso *et al.* 2005). When AMH is absent, the Müllerian duct is retained, but XY AMH<sup>-/-</sup> individuals are otherwise unambiguously male in appearance.

This created the impression that AMH had recently evolved to mediate limited male-specific functions. This perception of AMH has been rapidly changing. AMH has multiple faces. It appears to have both local (paracrine/autocrine) and circulatory (endocrine) roles in both sexes. The classical actions of AMH are paracrine (van Niekerk & Retief 1981, Mishina *et al.* 1996, Visser *et al.* 2007; Supplementary File, see section on supplementary data

given at the end of this article), but the identification of its actions outside of the reproductive tract is in its infancy. This review discusses AMH as a hormone and argues that the importance of AMH in the circulation has been overlooked, as it may primarily signal in concert with other members of the TGF $\beta$  superfamily (TGF $\beta$ -S). When operating in this mode, AMH may produce subtle variation in anatomical and physiological settings, which are only detectable by quantitative studies.

## AMH is a gonadal cytokine

AMH is synthesised in the gonads of all vertebrate species examined to date, including fish (Western *et al.* 1999, Halm *et al.* 2007), amphibians (Kodama *et al.* 2015), birds (Smith *et al.* 1999, Nishikimi *et al.* 2000), reptiles (Western *et al.* 1999, Shoemaker *et al.* 2007), marsupials (Juengel *et al.* 2002, Pask *et al.* 2004, 2010) and eutherian mammals (Josso 1973, Meyers-Wallen *et al.* 1987, Kuroda *et al.* 1990, Josso *et al.* 1993). In fish that do not have Müllerian ducts, AMH regulates the proliferation of germ cells in both sexes (Morinaga *et al.* 2007). In all vertebrates, the production of AMH is from the cells that nourish, protect and regulate the germ cells: the Sertoli cells of the testes and the ovarian granulosa cells. This suggests that AMH has ancient roles in phylogeny as a regulator of germ cells (Morinaga *et al.* 2007). AMH's role as an essential inducer of male sexual differentiation also predates the Müllerian duct. In some species of fish, AMH regulates the differentiation of the gonads into testes, and putatively determines testicular differentiation in birds (Smith & Sinclair 2004, Morinaga *et al.* 2007, Wu *et al.* 2010, Cutting *et al.* 2013). In mammals, AMH does not affect gonad determination as XY AMH<sup>-/-</sup> individuals have testes (Behringer *et al.* 1994, Josso *et al.* 2012), although AMH is required for the normal cellular and biochemical development of the mammalian testis (Mishina *et al.* 1996, Wu *et al.* 2005).

The study of AMH in lower vertebrates has centred on its role in gonadal differentiation, and there is little information available regarding the evolution of AMH as a hormone. In fish, the expression of the AMH-specific receptor (AMHR2) appears to be specific to the gonads (Morinaga *et al.* 2007, Kamiya *et al.* 2012), suggesting that AMH did not originally have hormonal action. However, AMH is in the blood of chicken embryos (Hutson & Donahoe 1983) as well as mammals (see below), suggesting that the endocrine functions of AMH are ancient.

Non-gonadal sites of AMH production have also been reported in fish and mammals (Wang *et al.* 2005, 2009a,

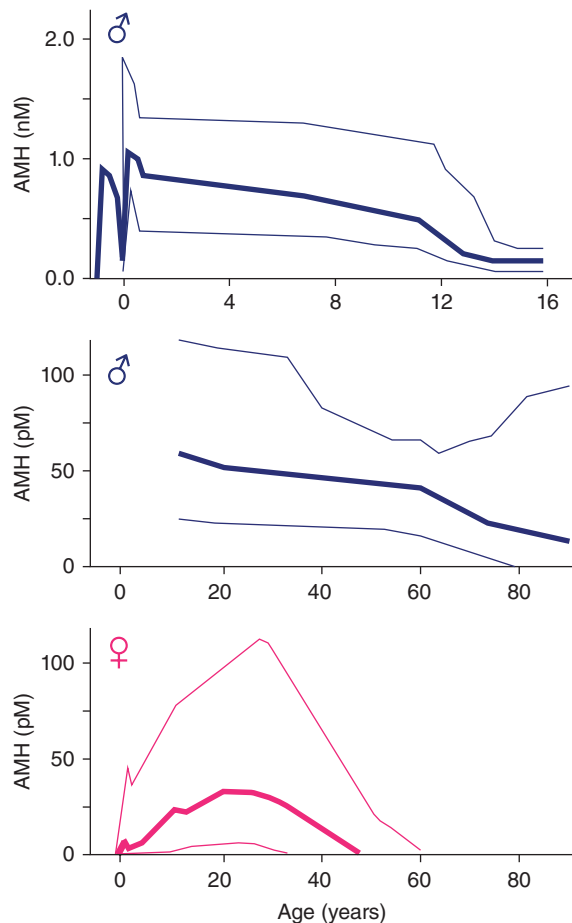
Halm *et al.* 2007, Ricci *et al.* 2010, Poonlaphdecha *et al.* 2011). No function has been ascribed to these sources of AMH, and the form of the AMH produced by non-gonadal cells has not been examined. In mammals at least, circulating AMH appears to be entirely derived from the gonads, as boys with anorchia lack AMH (Aksglæde *et al.* 2010) and because serum AMH is undetectable after either orchidectomy (Vigier *et al.* 1982) or oophorectomy (La Marca *et al.* 2005, Griesinger *et al.* 2012).

## Circulating levels of AMH are dimorphic

The levels of AMH in the circulation are broadly conserved between species, with the human pattern summarised in Fig. 1. In most, if not all, vertebrate species the secretion of AMH from the testis precedes the synthesis of testosterone and other gonadal hormones (Tran *et al.* 1987, Xavier & Allard 2003). The levels of AMH in immature males are high relative to those of adult males, and typically extend beyond the regression of the Müllerian duct (in species which have the duct). In mammals, the decrease in circulating AMH levels in males is associated with the pubertal transition. Ovarian production of AMH typically begins later in development, and the levels of AMH in the circulation of females never approach those of immature males. The levels of circulating AMH diminish as females age, and fall to very low levels during reproductive senescence. In post-menopausal women, AMH is typically undetectable. Consequently, AMH is variably male specific, strongly dimorphic with a male bias, or not dimorphic, depending on the stage of the life cycle (Fig. 1).

## Two forms of AMH exist in the circulation

The AMH gene encodes a theoretical 560 amino acid preproprotein, which is a member of the TGF $\beta$ -S (Cate *et al.* 1986). Cleavage of the amino signal peptide is thought to occur during protein synthesis, giving rise to proAMH (AMH<sub>25-560</sub>). ProAMH can be cleaved at position 451/452 to generate N- and C-terminus fragments (AMH<sub>N</sub> and AMH<sub>C</sub>; Cate *et al.* 1986), which remain associated as a stable non-covalently linked complex (AMH<sub>N,C</sub>) (Fig. 2; MacLaughlin *et al.* 1992, Wilson *et al.* 1993, Pankhurst & McLennan 2013). Circulating AMH has recently been discovered to contain a mixture of proAMH, which does not appear to activate AMHR2, and AMH<sub>N,C</sub>, which does (Pankhurst & McLennan 2013; see MacLaughlin *et al.* (1992) and di Clemente *et al.* (2010) for information relating to receptor binding). The implications of this are outlined in the paragraphs below.

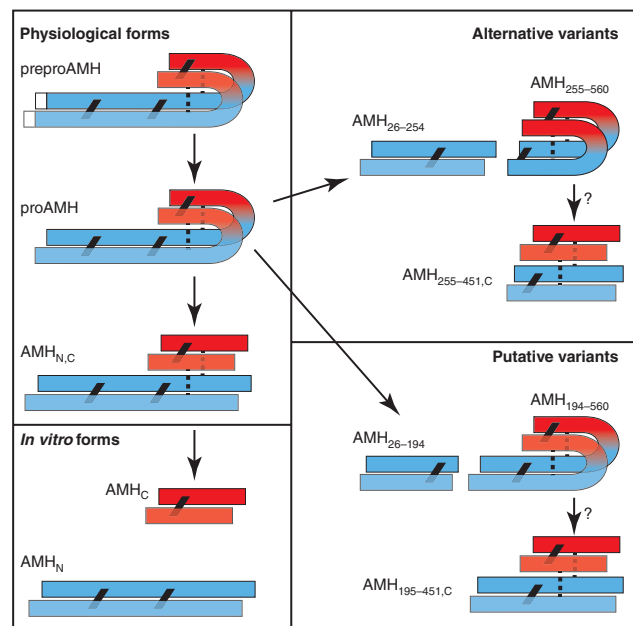
**Figure 1**

The levels of AMH in the human circulation vary during the life cycle, with a sexually dimorphic pattern. Boys (top panel) and men (middle panel) are presented separately, as the mean levels are so divergent. Females produce little or no AMH *in utero*. The thick lines illustrate the mean levels, and the thin lines the 95% CI. The illustration is based on the following references: Josso *et al.* (1993), Lee *et al.* (1996), Schwindt *et al.* (1997), Rajpert-De Meyts *et al.* (1999), Rey *et al.* (1999), Guibourdenche *et al.* (2003), Oppelt *et al.* (2005), Aksglaede *et al.* (2010), Hagen *et al.* (2010), Grinspon *et al.* (2011) and Chong *et al.* (2013). See also Tran *et al.* (1987), Munsterberg & Lovell-Badge (1991), Hirobe *et al.* (1992), Taketo *et al.* (1993) and Al-Attar *et al.* (1997) for information on mice and rats.

The C-terminal fragments of TGF $\beta$ -S ligands are receptor binding, but they tend to be relatively insoluble in physiological solutions. The formation of N- and C-terminal complexes increases solubility and facilitates the diffusion of TGF $\beta$ -S ligands through biological structures (Mueller & Nickel 2012). AMH conforms to this pattern with AMH<sub>C</sub> being receptor competent, with the presence of AMH<sub>N</sub> increasing its bioactivity (di Clemente *et al.* 1992, MacLaughlin *et al.* 1992, Wilson *et al.* 1993). Furthermore, the majority of loss-of-function mutations in human AMH are in the N-terminal domain,

emphasising that the non-receptor binding component of AMH has function (Josso *et al.* 2005). Free AMH<sub>C</sub> has yet to be detected in serum (Pankhurst & McLennan 2013), suggesting that AMH<sub>N,C</sub> only dissociates at or near the sites of AMH action (see also di Clemente *et al.* (2010)). Experimental studies of AMH have typically used rAMH<sub>C</sub>, which may not precisely or invariably mimic AMH<sub>N,C</sub>. Moving forward, we suggest that rAMH<sub>N,C</sub> should be preferentially used for the experimental analysis of AMH function.

AMH cleavage variants (AMH<sub>25-254</sub> and AMH<sub>255-560</sub>) occur *in vitro* when proAMH is cleaved with a serine protease, such as plasmin (Pepinsky *et al.* 1988; Fig. 2). AMH<sub>25-254</sub> and AMH<sub>255-560</sub> are not present at detectable levels in the blood of normal individuals (Pankhurst & McLennan 2013), but AMH fragments with these characteristics have been observed in the blood of a patient with a sex chord tumour (Ragin *et al.* 1992) and in equine granulosa cell tumours (Almeida *et al.* 2011). A putative cleavage site between amino acids 194/195 has been theorised based on sequence similarity with a cleavage site in Glass bottom boat, a *Drosophilla* TGF $\beta$ -S protein (Akiyama *et al.* 2012). AMH<sub>25-194</sub> has not been detected

**Figure 2**

Multiple forms of AMH are generated by post-translational cleavage. The amino acid sequence is numbered from the N-terminus of the preproAMH in this figure and in the text of this paper. Some sources number AMH from the N-terminus of the proAMH. When this numbering system is used, the alternatively cleaved AMH<sub>25-254</sub> will be numbered 1–230 and the putative forms will be numbered 1–170 and 171–427.

in physiological solutions, but naturally occurring mutations of the putative cleavage site in human AMH leads to persistent Müllerian duct syndrome (Imbeaud *et al.* 1994, Akiyama *et al.* 2012), the hallmark phenotype of XY AMH<sup>-/-</sup> individuals (Josso *et al.* 2012). This serves to emphasise that the form of AMH in the embryonic testes and in the circulation of prenatal males is unknown.

### ProAMH levels in the circulation vary

AMH ELISAs do not discriminate between proAMH and AMH<sub>N,C</sub> (Pankhurst *et al.* 2014). The historic information on circulating AMH is therefore an aggregate measure of two biologically distinct forms of AMH, one of which (proAMH) does not directly activate the canonical AMH receptors. We refer to this aggregate measure as total AMH.

When a prototype proAMH-specific ELISA is used, the ratio between proAMH and AMH<sub>N,C</sub> in the circulation varies between population groups. ProAMH is most abundant in boys, whereas AMH<sub>N,C</sub> is the predominant form in men and women. In all age groups, the ratio of proAMH to total AMH varies between individuals (McLennan & Pankhurst 2014). This raises questions about the physiological meaning of total AMH, as it has an uncertain relationship to the strength of receptor activation. In particular, we note that the pro- and mature forms of some cytokines activate different receptors (e.g. proNGF and NGF: Nykjaer *et al.* 2004, Hempstead 2014). Furthermore, some cytokine precursors can give rise to multiple distinct hormones through differential cleavage: for example, proopiomelanocortin is variably cleaved to ACTH,  $\alpha$ -,  $\beta$ -,  $\gamma$ -MSH,  $\beta$ -endorphin, CLIP,  $\beta$ - and  $\gamma$ -lipotropin (Takahashi & Mizusawa 2013). We therefore

argue against any presumption that proAMH has no biological activity, unless it is cleaved to AMH<sub>N,C</sub>.

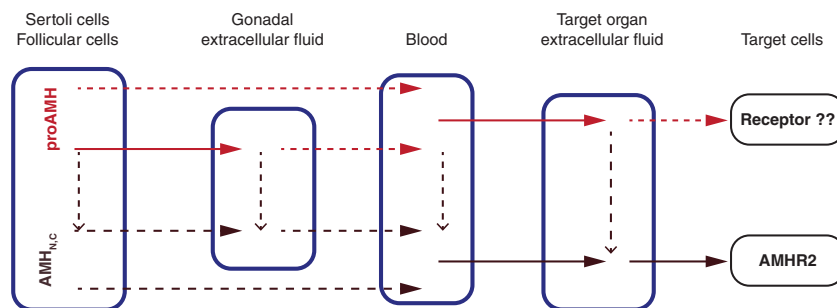
*A priori*, the relevant physiological measures of AMH may include the concentration of proAMH, the concentration of AMH<sub>N,C</sub> and/or the relative levels of proAMH and AMH<sub>N,C</sub>. The latter can be defined as the AMH prohormone index (API) = ((proAMH)/(total AMH) × 100). As the API decreases, the AMH in the circulation will have a greater ability to activate the canonical AMH pathway, without further processing (see below).

### Enzymatic cleavage of proAMH

Multiple enzymes cleave proAMH *in vitro* at position 254/255. These include proprotein convertases of the subtilisin/kexin-type 3 (PCSK3) (furin), PCSK5 (PC5 and PC6) and PCSK6 (PACE4) (Nachtigal & Ingraham 1996), which are PCSK. ProAMH is also cleaved by serine proteinases, most notably plasmin (Ragin *et al.* 1992). Plasmin dissolves fibrin blood clots, but it also has a proven role in the cleavage of the proforms of cytokines. Plasmin is synthesised as a larger precursor, plasminogen, which is activated by various proteases (plasminogen activators), whose activities in turn are regulated via activators and inhibitors (Ferraris & Sidenius 2013, Miles & Parmer 2013). The cleavage of proAMH may therefore be subject to complex regulation *in vivo*, although this remains to be proven.

### Gonadal cleavage of proAMH

The API may reflect multiple gonadal influences (Fig. 3). Sertoli and granulosa cells express enzymes that cleave proAMH, with the levels of the enzymes and/or their



**Figure 3**

The route between AMH synthesis and receptor activation. The confirmed (solid arrows) and putative (dotted arrows) pathway for proAMH transport and cleavage (side arrows) are illustrated in red. The flow of AMH<sub>N,C</sub> is illustrated with brown arrows. The figure only illustrates the pathways for

endocrine signaling. Sertoli cells release additional AMH into the lumen of the seminiferous tubules, with this AMH being incorporated into seminal plasma. A proportion of the AMH in the ovarian follicular fluids may be released into the uterine tube during ovulation.

regulators varying during testicular (Nachtigal & Ingraham 1996, Guo *et al.* 2007, Le Magueresse-Battistoni 2007, Uhrin *et al.* 2007) and ovarian (Bae *et al.* 2008) development, the seminiferous cycle (Guo *et al.* 2007, Le Magueresse-Battistoni 2007, Uhrin *et al.* 2007), the ovarian cycle (Bae *et al.* 2008, Wang *et al.* 2014), the stage of ovarian follicular development (Ohnishi *et al.* 2005, Bae *et al.* 2008, Antenos *et al.* 2011) and during pregnancy (Kwok *et al.* 2013). However, the AMH in ovine follicular fluid is predominantly proAMH with little AMH<sub>N,C</sub> (Campbell *et al.* 2012), suggesting that AMH can be synthesised and released with little or no prior cleavage.

The enzymes that cleave proAMH have extracellular forms (Seidah *et al.* 2006). The form of AMH within the gonads and within the circulation may therefore depend on the route AMH has taken to reach its current location. For example, the thecal cells of ovarian follicles usually have higher levels of PCSK3 and PCSK5 than do the adjacent granulosa cells, with these levels under gonadotrophin regulation (Bae *et al.* 2008, Kelty & Curry 2010). This may alter the form of AMH if the AMH diffuses through the thecal layer. Similarly, AMH produced at one site of the seminiferous tubules or by one ovarian follicle may be cleaved by other parts of the gonad. A key issue here is whether or not the AMH in the circulation is distinct from the AMH that acts as a paracrine regulator of the gonads. Similarly, AMH is present in semen (Fallat *et al.* 1996, Fenichel *et al.* 1999), but it is also unclear whether seminal and circulating AMH are from a common pool.

### Extra-gonadal cleavage of proAMH

Recombinant proAMH induces regression of the Müllerian duct in organ culture, leading to speculation last century that target tissues can process proAMH (Cate *et al.* 1986, Wilson *et al.* 1993). This idea has been in abeyance, but has substantial merit as the putative cleavage enzymes are widely expressed (Villeneuve *et al.* 1999, Stawowy *et al.* 2001, Cain *et al.* 2003, Veinot *et al.* 2004), under the influence of various physiological and pathological stimuli (Stawowy *et al.* 2001, Veinot *et al.* 2004, Marchesi *et al.* 2011). Under this circumstance, the AMH<sub>N,C</sub> in circulation defines a basal level of activation, which can be amplified by the local cleavage of proAMH to AMH<sub>N,C</sub>. This is mechanistically similar to testosterone signalling, where activation of the androgen receptor is influenced by circulating levels of testosterone, and by the local conversion of testosterone to the more potent dihydrotestosterone.

Recombinant proAMH is not readily cleaved in human serum *in vitro* (Pankhurst *et al.* 2014). However, proAMH-cleaving enzymes are found in vascular tissues (Stawowy *et al.* 2001, Veinot *et al.* 2004), and it is therefore possible that proAMH is cleaved to AMH<sub>N,C</sub> whilst in the circulation. Equally, the cardiovascular system is a putative target for circulating AMH (Appt *et al.* 2012, Dennis *et al.* 2013, Yarde *et al.* 2014), and any cleavage by vascular sources may only have a local effect.

### The hallmark function of AMH may be atypical

AMH is a phylogenetically ancient protein, whose levels in the circulation are subject to complex regulation during the life cycle in a sexually dimorphic manner (reviewed above). The forms of AMH in the circulation are regulated, with the regulation potentially being very complex (reviewed above). The existence of AMH was detected 99 years ago (Lillie 1916). Despite this, the endocrine functions of AMH are largely unknown. This suggests that the endocrine actions of AMH are cryptic and are being obscured by a misconception(s) about the biology of AMH. Canonically, AMH is an atypical TGFβ-S ligand, which signals in isolation of other members of its family. We alternatively suggest that AMH may be a typical TGFβ-S ligand, with the regression of the Müllerian duct being an atypical action for AMH.

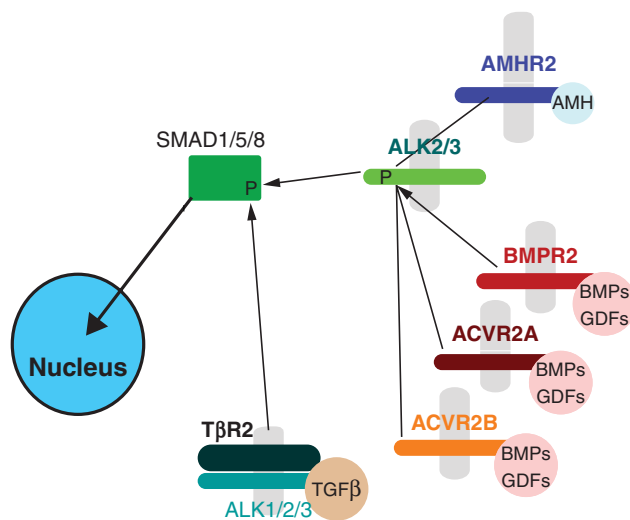
### TGFβ-S ligands signal interactively

The TGFβ-S ligands signal through complexes consisting of type 1 and type 2 receptors. There are over 30 mammalian TGFβ-S ligands, which share five type 2 receptors and seven type 1 receptors. Consequently, TGFβ-S signalling typically arises from the interactions between multiple ligands and multiple receptors (Shi & Massague 2003, Moustakas & Heldin 2009). One of the five type 2 receptors (AMHR2) is AMH specific, which is in stark contrast to the superfamily as a whole, which shares the other four receptors. This argument is supported by the observation that AMH<sup>-/-</sup> and AMHR2<sup>-/-</sup> XY individuals have the same overt phenotype, the persistence of the Müllerian duct (Behringer *et al.* 1994, Jamin *et al.* 2002, Josso *et al.* 2005). This is very strong evidence that AMH induces regression of the Müllerian duct via AMHR2, independently of other TGFβ-S ligands. However, this does not prove that AMH invariably signals as an isolated regulator, or that AMHR2 is essential for AMH (AMH<sub>N,C</sub> or proAM) signalling elsewhere in the body. As argued below, AMH may primarily signal in co-operation

with other TGF $\beta$ -S ligands. Similarly, this evidence does not exclude the possibility that proAMH is able to signal via an unidentified receptor. For example, NGF has a one-to-one relationship with TrkA and the phenotypes of NGF<sup>-/-</sup> and TrkA<sup>-/-</sup> mice were thought to be identical (Carroll *et al.* 1992, Smeyne *et al.* 1994) before Sortilin was identified as a proNGF receptor (Nykjaer *et al.* 2004).

The canonical function of the type 2 receptors is to activate type 1 receptors, which initiate the downstream signalling (Shi & Massague 2003, Moustakas & Heldin 2009). AMH does not have a unique type 1 receptor, and to date, there is no evidence for the existence of an AMH-specific intracellular pathway. The AMH-induced regression of the Müllerian duct is mediated by two type 1 receptors (BMPR1A/ALK3 and ACVR1/ALK2), with functional redundancy between the receptors (Orvis *et al.* 2008). The bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) also use these receptors, with the BMP/GDFs constituting more than half of the TGF $\beta$ -S (Shi & Massague 2003, Mueller & Nickel 2012). The three BMP/GDF type 1 receptors activate the SMAD1/5/8 intracellular pathway, which also transduces a proportion of the TGF $\beta$  subfamily (Shi & Massague 2003, Moustakas & Heldin 2009; Fig. 4).

Mice with null-mutations of TGF $\beta$ -S ligands frequently exhibit limited overt phenotypes (Kingsley *et al.* 1992, Shull *et al.* 1992, Kaartinen *et al.* 1995, Settle *et al.* 2003),



**Figure 4**  
AMH signaling involves elements that are common to other TGF $\beta$ -S ligands. The type 1 receptors used by AMH are shared with the BMP and GDF ligands via two type 2 receptors. AMH, BMP and GDF ligands activate the SMAD1/5/8 pathway, along with a minor portion of TGF $\beta$  signalling (based on Shi & Massague (2003) and Moustakas & Heldin (2009)).

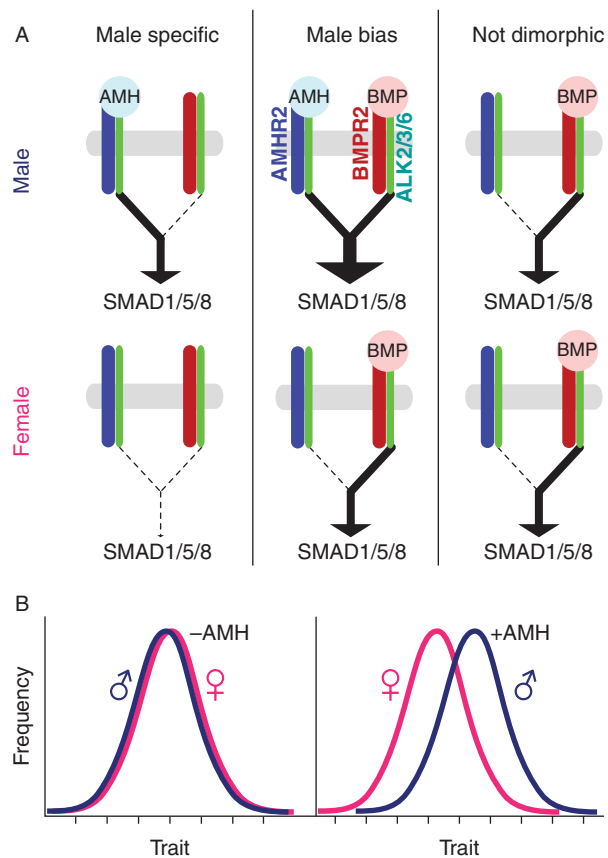
with the full spectrum of their biology only emerging when multiple ligands (Settle *et al.* 2003, Tilleman *et al.* 2010) and/or receptors are absent (Oshima *et al.* 1996, Beppu *et al.* 2000). Non-redundant signalling appears to occur when a single TGF $\beta$ -S ligand is predominantly or totally activating the common downstream cascade, although non-canonical signalling (Massague 2012) and/or other mechanisms may also contribute to this phenomenon (Mueller & Nickel 2012). The BMP/GDF ligands bind promiscuously and can activate one or more of three type 2 receptors: BMPR2, ACVR2A and ACVR2b (Shi & Massague 2003, Mueller & Nickel 2012). The BMP/GDF ligands and their type 2 receptors are very broadly expressed (Feijen *et al.* 1994, Lowery & de Caestecker 2010, Miyazono *et al.* 2010). The full pattern of AMHR2 expression is still only partially elucidated (see below), but it is possible that the only place where the combination of AMH and AMHR2 is solely activating the SMAD1/5/8 pathway is the Müllerian duct.

### AMH may generate sex biases during mammalian development

When a developing cell expresses AMHR2 and BMPR2, the SMAD1/5/8 pathway would be activated in both sexes via BMPR2, with AMH inducing a male-specific augmentation of this pathway (Fig. 5). *A priori*, the extent of the sex bias should relate to the relative levels of AMH, BMPs, AMHR2, BMPR2 and associated signalling molecules. If so, AMH may generate a small male bias at one site and large bias at a different location, depending on the local concentrations of BMPs and BMPR2. A similar argument can be made with respect to the other type 2 receptors (Act2R and Act2RB) and ligands which contribute to the activation of the SMAD1/5/8 pathway.

The BMPs, GDFs and the activins contribute to the generation of the basic body plan during embryogenesis, after which they influence the development of most organs, including the brain (Liu & Niswander 2005, Miyazono *et al.* 2005, Park *et al.* 2006, Moustakas & Heldin 2009, Lowery & de Caestecker 2010). Consequently, AMH would be expected to produce a male-specific augmentation to numerous developmental processes. This would be expected to induce sex biases, but would not be expected to induce sex-specific biology. Sex biases primarily relate to reproductive fitness.

The biology of sex differences has historically been concerned with the ability to procreate, which requires appropriate sex organs and an ability to recognise the opposite sex. Reproductive fitness extends beyond this.

**Figure 5**

AMH and BMP can combine to produce sex differences. (A) When AMH signals alone (left panel), the SMAD1/5/8 pathway is male specific; when AMH and BMP both signal (middle panel), the SMAD1/5/8 pathway has a male bias; when BMP signals alone, the SMAD1/5/8 pathway is not dimorphic. (B) The left panel illustrates the population distribution for a hypothetical trait under BMP control. The right panel illustrates the hypothetical population distribution produced by AMH and BMP in combination.

It includes subtle quantitative variations in physical and behavioural traits that enhance an individual's ability to compete with other members of their sex. Quantitative sex biases are present in most organs and are common in behaviour, but the bias is typically sufficiently small for the female and male ranges to overlap. Consequently, non-reproductive tissues do not have distinct male or female form, with their characteristics only being sexually dimorphic at the level of the population (when groups of women and men are compared). For example, women on average are shorter than men on average, but the sex of tall women and short men is unambiguous. That is, any deficiency in sex biases would be expected to affect fitness, but is unlikely to alter the perception of the person's sex.

The phenotype of XY  $AMH^{-/-}$  and  $AMHR2^{-/-}$  mice is consistent with the above mechanism. For example, spinal motor neurons express  $BMPR2$  and  $AMHR2$ , with  $AMH$  and  $BMP6$  promoting the survival of motor neurons *in vitro* (Wang *et al.* 2005, 2007). The number of neurons in the brain is regulated through the control of programmed cell death (Oppenheim 1991). Hence,  $AMH$  should induce a male bias in the number of motor neurons. Consistent with this, WT male mice have 16% more spinal motor neurons than their WT sisters, with this sexual dimorphism being absent in  $AMH^{-/-}$  and  $AMHR2^{-/-}$  mice (Wang *et al.* 2009b).  $AMH^{+/-}$  mice exhibit a male bias which is half that of their WT brothers (Wang *et al.* 2009b). Similar  $AMH$ -dependent sex biases have been detected in the numbers of cerebella Purkinje cells (Wittmann & McLennan 2011) and in the calbindin<sup>+ve</sup> neurons of the sexually dimorphic nucleus of the preoptic area (Wittmann & McLennan 2013a) and the bed nucleus of stria terminalis (Wittmann & McLennan 2013b). The latter two brain nuclei are highly dimorphic in adults, with  $AMH$  being only responsible for the sex differences that develop before the onset of puberty (Wittmann & McLennan 2013a,b). This serves to emphasise that the differences between the sexes is a product of multiple mechanisms (Arnold 2004), of which  $AMH$  is but one.

Male  $AMH^{-/-}$  mice prefer to sniff female rather than male bedding, suggesting that they are heterosexual (Wittmann & McLennan 2013b). However,  $AMH^{-/-}$  male pups exhibit female-like exploration of novel objects (Morgan *et al.* 2011a), and  $AMH^{-/-}$  adult mice exhibit female-like behaviours when exploring a chamber (Wang *et al.* 2009b). Both of these behaviours are thought to relate to the propensity of male mice to hold larger territories than female mice, which is a component of reproductive fitness.

Null mutations of  $AMH$  and  $AMHR2$  are very rare in humans, and detailed studies of their characteristics are not available. However, putative functions for  $AMH$  in humans can be detected by correlating the traits of boys with their levels of  $AMH$ . This approach is based on the observations that  $AMH$  levels of age-matched boys show high inter-person variation, with this variation being stable over time (Akslaede *et al.* 2010, Morgan *et al.* 2011b). When this approach is used, the level of a boy's  $AMH$  negatively correlates with indexes of his physical (Morgan *et al.* 2011b) and cognitive development (Morgan *et al.* 2011c). Boys tend to be less mature than age-matched girls across many traits, and the observed correlations suggest that testicular  $AMH$  contributes to this by slowing the speed at which males develop. It is not currently

possible to directly test causality here. However, if AMH does slow the rate of human development, then biological theory suggests that boys with high levels of AMH should be less affected by developmental disorders than boys with lower levels of AMH. Consistent with this *a priori* prediction, the severity of symptoms of boys with an autistic spectrum disorder negatively correlates with their levels of circulating AMH (Pankhurst & McLennan 2012).

In summary, AMH is an ancient inducer of the male phenotype. It can induce male-specific biology (absence of a uterus), but in mammals the overt male-specific features are mainly the province of testosterone. The predominant role of AMH may be to interact with other TGF $\beta$ -S ligands to produce numerous sex biases throughout the body. The initial murine and human data are consistent with this hypothesis, but the available data are very limited. The role of AMH in shaping the characteristics of boys, and the resulting men, remains largely unexplored.

### AMH can signal at adult circulatory levels

AMH, like other TGF $\beta$ -S ligands, has context-dependent dose–response curves. Consequently, the fact that adult men have an order-of-magnitude less circulating AMH than boys do (Fig. 1) is not an *a priori* reason to presume that AMH is not a hormone in adults. Embryonic neurons *in vitro* exhibit a log-linear dose curve, with adult-like levels of AMH producing biologically significant effects (Wang *et al.* 2005). Embryonic neurons are an order-of-magnitude more sensitive to AMH than some cell lines (compare Wang *et al.* (2005) and Masiakos *et al.* (1999) and Pieretti-Vanmarcke *et al.* (2006)). The determinants of the dose–response curve for AMH are unknown, but may include the ability of the target cells to cleave proAMH adjacent to the receptor, as the recombinant AMH used in most experiments is a mixture of proAMH and AMH<sub>N,C</sub>.

The specificity and dose–response curves of the TGF $\beta$ -S are a product of binding proteins as well as receptors. The ligand-specificities of the binding proteins can be distinct from those of the receptors, creating interactions between the various TGF $\beta$  subfamilies (Shi & Massague 2003, Massague 2012). The AMH-induced regression of the Müllerian duct does not require a binding protein, and the influence of binding proteins on AMH signalling has therefore not been thoroughly examined. Preliminary results indicate that the follistatins increase the response of reporter cells to AMH<sub>N,C</sub> (Kawagishi *et al.* 2014). Follistatins are classical inhibitors of the activins (de Kretser *et al.* 2004, Hedger & de Kretser 2013), which

activate the SMAD2/3 intracellular pathway (Shi & Massague 2003). Consequently, when follistatins are present, the balance between AMH-induced activation of SMAD1/5/8 and activin-induced activation of SMAD2/3 may be altered, although *in vivo* work is needed to prove this. Most importantly, the preliminary observation with follistatin re-enforces the notion that AMH is a typical TGF $\beta$ -S ligand, and its signalling is therefore context-dependent.

The context dependency of TGF $\beta$ -S signalling has multiple causes, one of which is competition between ligands and receptors (Massague 2000, 2012). Consequently, the relative levels of receptors can be important, which leads to yet another unresolved paradox of AMH. The AMH-specific receptor (AMHR2) is the most abundant cytokine receptor in motor neurons, with levels that are much higher than other type 2 receptors and the type 1 receptors. Despite this, the neuronal-levels of AMHR2 are orders-of-magnitude less than occurs in the testes, ovary and Müllerian duct (Wang *et al.* 2005). The physiological significance of this observation is unclear.

The original studies of the distribution of AMHR2 were set to detect gonad-like levels of AMHR2. This created the false impression that AMH only signalled in the gonads and its associated tissues. The presence of AMHR2 has been rigorously proven in multiple sites, including the nervous system, lungs, mammary glands, uterus and prostate (Catlin *et al.* 1997, Segev *et al.* 2000, 2001, 2002, Hoshiya *et al.* 2003, Renaud *et al.* 2005, Wang *et al.* 2005, 2009a,b, Lebeurrier *et al.* 2008). Similarly, broad expression is detected in mice with a reporter gene driven by the endogenous *Amhr2* promoter (AMHR2-Cre-lacZ: Wang *et al.* 2009b) (IS McLennan & Dennis NA, unpublished observations). However, the rigorous detection of the cellular location of AMHR2 is currently a limiting problem, as the available anti-AMHR2 antibodies show cross-reactivity in some but not all tissues.

### Circulatory AMH may signal reproductive status in adults

The physiology of circulating AMH in adults is an unwritten book, for which there is not even the briefest outline of the plot. As outlined above, the historic evidence suggested that circulating AMH levels in adults could not signal. The current evidence reverses this situation: AMH is present in the circulation of adults, at levels sufficient to activate its receptors, and its receptors appear to be broadly expressed. One thing is certain: AMH is not vital in the way that classical hormones are, as



AMH<sup>-/-</sup> individuals do not show any gross symptoms. However, if AMH signals in the way described above, then symptoms would not be expected. In adults, the TGFβ-S regulates multiple aspects of homeostasis, through a process that integrates multiple signals. By doing so, the TGFβ-S helps to ensure that the functions of cells reflect both their immediate environment and the status of the body as a whole. The function of AMH in adults may be to add a gonadal influence to this integrative process.

The gonads release multiple hormones in both sexes, which in general signal through different intracellular cascades. The activins and the inhibins are TGFβ-S ligands, which predominantly signal through the SMAD2/3 pathway, whereas the sex steroids and INSL3 signal through other pathways. *A priori*, it is therefore possible that each of these hormones transmits the same biological information but to different parts of the intracellular cascade. However, the levels of circulating AMH in men show almost no concordance with the levels of the other testicular hormones. This suggests that AMH may transmit different information about the gonads than testosterone and the other testicular protein hormones. The number and state of the germ cells in the gonads is the major determinant of the level of AMH in the circulation. This is most clearly established for females (Visser *et al.* 2012), but it may also be the case for males, as germ cells regulate Sertoli cells (O'Shaughnessy *et al.* 2008, Cool *et al.* 2012, Dabaja *et al.* 2015). Consequently, AMH is uniquely and ideally placed to convey information about the current and future reproductive capacity of an individual. At this stage, it is difficult to predict how this translates into AMH-mediated changes in the properties of cells.

If AMH is part of BMP signalling, then one way forward is to examine sites of proven BMP regulation. The BMPs are broad regulators of the cardiovascular system, with some BMP ligands being putative cardiovascular hormones (Lowery & de Caestecker 2010). Initial evidence is consistent with the cardiovascular systems as being a target tissue for AMH. The levels of circulating AMH in men associate with the size of their aorta (Dennis *et al.* 2013). AMH levels on average differ between men with defined cardiovascular conditions (Dennis *et al.* 2013), with AMH also being linked to pregnancy-associated hypertension (Shand *et al.* 2014). In female rhesus monkeys, premenopausal AMH levels associate with subsequent atherosclerosis (Appt *et al.* 2012). However, to date, there has been no experimental examination of whether AMH can directly influence any cardiovascular parameter.

## Conclusion

AMH is a hormone/cytokine that has been trapped by its name and the history of its discovery. If AMH is viewed as the gonadal BMP, it changes from being an atypical cytokine with a few specialised functions to a broad pleiotropic regulator which changes with the stage of life. Initially it may create diversity within the male lineage. As mammals transition into adulthood, AMH may become a signal to the body about the capacity/characteristics of the gonads, with this signal being of relevance to both sexes.

### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0206>.

### Declaration of interest

I S M and M W P have IP relating to immunoassays that detect specific forms of AMH.

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