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REVIEW ARTICLE

Depolarising and Hyperpolarising Actions of $GABA_A$ Receptor Activation on Gonadotrophin-Releasing Hormone Neurones: Towards an Emerging Consensus

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The gonadotrophin-releasing hormone (GnRH) neurones represent the final output neurones of a complex neuronal network that controls fertility. It is now appreciated that GABAergic neurones within this network provide an important regulatory influence on GnRH neurones. However, the consequences of direct $GABA_A$ receptor activation on adult GnRH neurones have been controversial for nearly a decade now, with both hyperpolarising and depolarising effects being reported. This review provides: (i) an overview of $GABA_A$ receptor function and its investigation using electrophysiological approaches and (ii) re-examines the past and present results relating to GABAergic regulation of the GnRH neurone, with a focus on mouse brain slice data. Although it remains difficult to reconcile the results of the early studies, there is a growing consensus that GABA can act through the $GABA_A$ receptor to exert both depolarising and hyperpolarising effects on GnRH neurones. The most recent studies examining the effects of endogenous GABA release on GnRH neurones indicate that the predominant action is that of excitation. However, we are still far from a complete understanding of the effects of $GABA_A$ receptor activation upon GnRH neurones. We argue that this will require not only a better understanding of chloride ion homeostasis in individual GnRH neurones, and within subcellular compartments of the GnRH neurone, but also a more integrative view of how multiple neurotransmitters, neuromodulators and intrinsic conductances act together to regulate the activity of these important cells.

Key words: GABA, chloride cotransporter, electrophysiology.

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The amino acid neurotransmitter GABA has long been recognised as being of prime importance in the control of GnRH release and thereby gonadotrophin secretion in mammals. Indeed, the first report of GABA modulation of luteinising hormone (LH) secretion was published in *Science* in 1974 (1) and a PubMed search of 'GABA & GnRH' presently generates a list of 413 publications. A large number of *in vivo* intracerebral and i.c.v. studies now support an important role for GABA in the control of many aspects of gonadotrophin secretion, and in a variety of species (2). The prevailing view from these *in vivo* studies has been that GABA acts through $GABA_A$ receptors to suppress both pulsatile and surge modes of LH secretion (2), although there are also examples of stimulatory actions of GABA action on GnRH/LH release (3, 4). However, the precise mechanisms and loci of GABA action within the GnRH neuronal network (i.e. GnRH neurones, afferent neurones and associated glial cells), remain unclear. Studies examining the effects of GABA on GnRH neurones at a cellular level have progressed from GT1 cell and embryonic nasal placode models through to brain slice work in transgenic mice. Experiments in the latter preparation have enabled the actions of GABA to be examined on GnRH neurones closer to the *in situ* situation. This review intends to provide a background to understanding $GABA_A$ receptor function and experimental interpretation, and to address the controversy that has arisen regarding the effects of direct $GABA_A$ receptor activation on adult GnRH neurones.

$GABA_A$ receptor function

Types of GABA receptors

GABA binds to two classes of receptors: $GABA_A$ and $GABA_B$, with a third class, $GABA_C$, being considered by some to be a subclass of the $GABA_A$ group with similar structure and function (5, 6). $GABA_A$ and $GABA_B$ receptors can be located on the pre- and postsynaptic membrane, and also outside the synapse at extrasynaptic locations (Fig. 1).

Although this review focuses upon the $GABA_A$ receptor, it is important to recognise that $GABA_B$ receptors are also likely to play a role in the direct GABAergic modulation of GnRH neurone excitability (7). $GABA_B$ receptors are metabotropic seven-transmembrane domain G-protein-coupled receptors that regulate downstream channels (8). It is worth emphasising that the $GABA_B$ receptor itself is not an ion channel with changes in membrane current generated in response to $GABA_B$ receptor activation being a result of receptor-mediated changes in downstream effector channels. Typically, the postsynaptic $GABA_B$ receptor is coupled to potassium channels, whereas the presynaptic $GABA_B$ receptor is linked to calcium channels (8). Although information remains scarce, this is likely to be the situation for GnRH neurones (Fig. 1). Stimulation of $GABA_B$ receptors results in the activation of a specific class of potassium channels that generates membrane hyperpolarisation and inhibition of GnRH neurone firing (7). Preliminary data also indicate that calcium channel-linked $GABA_B$ receptors are present on GABAergic nerve terminals regulating GnRH neurone excitability (X. Liu & A. E. Herbison, unpublished data). Although there is some evidence that $GABA_B$ receptors can act to suppress high-frequency activated GABA inputs to GnRH neurones (9), the roles of endogenous GABA in activating $GABA_B$ receptors on GnRH neurones remain largely unknown.

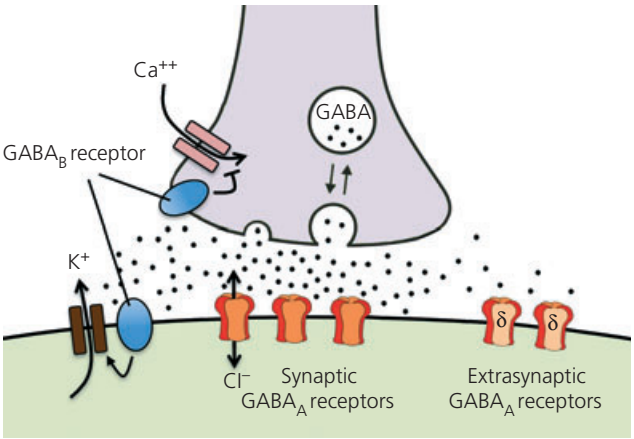

Fig. 1. Schematic representation of GABA signalling at the gonadotrophin-releasing hormone (GnRH) neurone in the mouse. GABA terminals (grey) synapsing on GnRH neurones (green) are likely to have $GABA_B$ receptors that act to inhibit calcium entry to the terminal, thereby suppressing presynaptic activity. GABA released from the nerve terminal activates postsynaptic $GABA_A$ receptors within the synapse to generate a fast phasic depolarising or hyperpolarising response and also $GABA_B$ receptors that activate potassium channels that hyperpolarise the membrane. Spill-over of GABA from the synapse activates extrasynaptic $GABA_A$ receptors (expressing the delta receptor subunit) that provide a low level tonic influence on membrane polarisation.

The $GABA_A$ receptor is a ligand-gated ion channel composed of five subunits (typically 2α, 2ß plus a variable fifth), each of which contributes to a central pore (10). Binding of two GABA molecules at the α–ß interfaces most effectively gates (i.e. opens) the channel (11), although both single ligand and ligand-independent opening are possible (12, 13). The $GABA_A$ receptor pore is permeable in both directions to monovalent anions, the most physiologically relevant of which are Cl^- and HCO_3^- (14, 15). A variety of $GABA_A$ receptor subunits are expressed in GnRH neurones (see below), functional $GABA_A$ receptor-mediated currents can be measured in response to both exogenous and endogenous GABA (16–18), and spontaneous GABAergic transmission is altered in different reproductive conditions (see below). Thus an understanding of the consequences of $GABA_A$ receptor activation is important in forming a complete picture of GnRH neurobiology.

The opening of the intrinsic ion channel of the $GABA_A$ receptor is neither innately excitatory, nor inhibitory to a cell (10, 19). Rather, as with any ion channel, the net current flow through the pore depends primarily on two variables: the concentration gradients of the permeable anions and the membrane potential of the cell at the time the channel opens. These forces define the electrochemical potential that drives ion flow through the open channel. The membrane potential at which these two forces are equal and opposite is referred to as the reversal potential for the current through that class of channels; at this potential, there is no net current flow.

As noted above, both Cl^- and HCO_3^- can flow through the $GABA_A$ receptor pore. The permeability of $GABA_A$ receptors to Cl^- is typically two to five-fold greater than that to HCO_3^- (14, 20) in mammalian cells. Under physiological conditions, Cl^- is the main charge carrier through the $GABA_A$ receptor. However, HCO_3^- can also contribute to net current via the $GABA_A$ receptor depending on both pH and intracellular $[Cl^-]$ (21, 22). Hence, although dominated by Cl^-, the reversal potential for the $GABA_A$ receptor (E_{GABA}) results from the flow of both Cl^- and HCO_3^- ions. Intracellular bicarbonate levels tend to accumulate because of the action of carbonic anhydrase to generate HCO_3^- from CO_2 and H_2O (23). As the reversal potential for HCO_3^- is quite depolarised, E_{GABA} is slightly more depolarised than the reversal potential for Cl^- (E_{Cl}), alone. Importantly, as shown below, E_{Cl} can vary from cell to cell and with developmental stage, and this results in concomitant variations in E_{GABA}.

Cation chloride co-transporters

The intracellular chloride concentration of a cell is essentially set by the action of cation chloride co-transporters (Fig. 2). These transporters are secondary active transporters that utilise the driving force in Na^+ and K^+ gradients established by the Na-K ATPase (19). The chloride-cation co-transporters are electrically neutral; thus, their rate of transport does not contribute in and of itself to membrane potential. There are two primary classes: K-Cl co-transporters (KCCs) and Na-K-Cl co-transporters (NKCCs). Under physiological

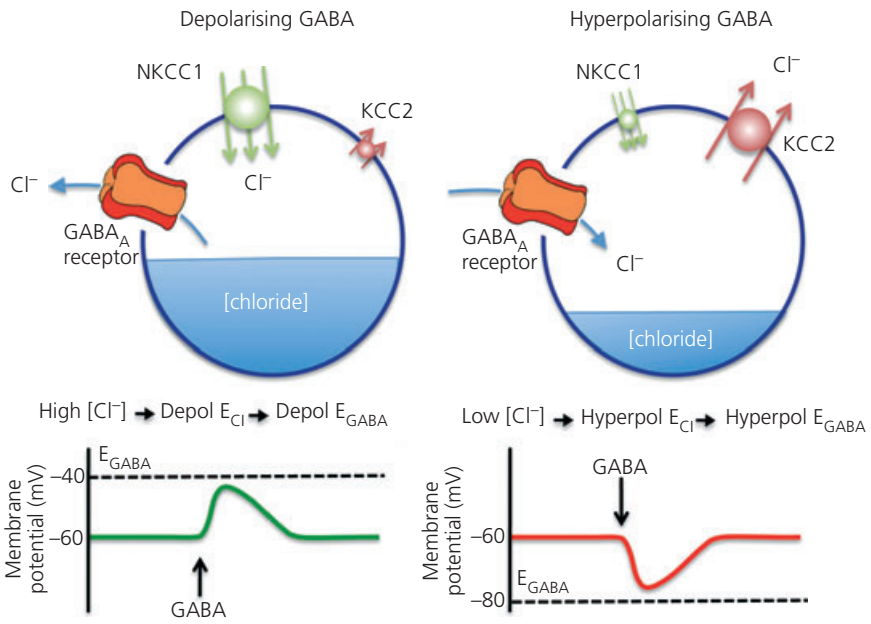

Fig. 2. Schematic diagram showing the typical interrelationships between chloride transporters, chloride levels and $GABA_A$ receptor function that underlie depolarising and hyperpolarising actions of GABA in neurones. The balance of chloride ion transport into the neurone determines the intracellular chloride ion concentration. In neurones under physiological conditions, this is dependent principally on the activity of the sodium-potassium-chloride co-transporter 1 (NKCC1), bring chloride into the cell, and the potassium-chloride co-transporter 2 (KCC2), removing chloride from the cell. Cells with a relatively high intracellular chloride ion concentration will have a relatively depolarised reversal potential for chloride (depol E_{Cl}) that, in turn, sets a relatively depolarised reversal potential for GABA (depol E_{GABA}), in this case imagined to be −40 mV. When the cell is exposed to GABA, the $GABA_A$ receptor will open and the cell membrane potential will move towards E_{GABA} as chloride ions leave the cell, thus generating a depolarising response. The opposite pattern of events occurs for cells with a relatively low intracellular chloride concentration resulting in hyperpolarisation (right). Note that a level of intracellular chloride could be attained at which E_{GABA} is similar to the resting membrane potential (imagined here to be −60 mV), in which case activation of the $GABA_A$ receptor would have minimal effects on membrane potential. Adapted with permission (138).

conditions, KCCs extrude Cl^- from the cell, lowering intracellular Cl^- levels. By contrast, NKCCs accumulate Cl^- and raise intracellular Cl^- levels (Fig. 2).

The intracellular Cl^- concentration in most neurones is reduced from elevated to low levels during early postnatal life. This shift has been associated with an increase in KCC expression and function, in particular KCC2B, concomitant with a reduction in NKCC1 (24) (Fig. 2). Although this neonatal developmental shift has become somewhat dogma, cell types are still being identified that do not follow this developmental pattern, with several cell types continuing to exhibit elevated intracellular Cl^- concentrations either locally or globally into adult life (25–27). For example, primary olfactory neurones maintain elevated intracellular Cl^- throughout adult life as a result of the continued function of NKCC1 and low expression of KCC2 (28, 29). In addition to global expression patterns, post-translational modification and subcellular location of cation chloride transporters appear to be critical to sculpting response to GABA. For example, NKCCs are activated by phosphorylation, whereas KCCs can be inhibited or activated by phosphorylation (30–33), and NKCC1 may be targeted to the axon initial segment where axon potentials are initiated (34, 35). Thus, KCCs and NKCCs set intracellular Cl^- concentrations in a cell-type- and subcellular location-specific manner and this, in turn, provides the dominant force setting E_{GABA} of the cell/region (Fig. 2).

Consequence of $GABA_A$ receptor activation

Whether activation of $GABA_A$ receptors results in depolarisation or hyperpolarisation of a neurone's membrane potential depends on the relationship of E_{GABA} to the membrane potential of the cell at the time the receptor is gated by GABA (Fig. 2). When the membrane potential of a cell is depolarised relative to E_{GABA}, opening the intrinsic channel of the $GABA_A$ receptor will hyperpolarise the membrane; similarly, when the membrane potential is hyperpolarised relative to E_{GABA}, opening this channel will depolarise the membrane (Fig. 2). In other words, if the membrane potential of a cell was −60 mV and E_{GABA} was −40 mV, then opening the $GABA_A$ receptor would result in an outward flow of Cl^- in an attempt to depolarise the cell to a membrane potential of −40 mV. If E_{GABA} was −80 mV, then $GABA_A$ receptor activation would result in Cl^- entering the cell to hyperpolarise it. Hence, it can be seen that E_{GABA} is critical to determining whether GABA will depolarise or hyperpolarise a cell. The relationship of E_{GABA} to action potential threshold is also important; if E_{GABA} is more depolarised than threshold, activation of $GABA_A$ receptors can depolarise a cell sufficiently to initiate action potential firing. As noted above, E_{GABA} is determined primarily by E_{Cl}, which is established by the activity of the chloride ion co-transporters (Fig. 2). This is why the neonatal developmental changes in NKCCs and KCCs noted above are so fundamental to the shift from depolarising to

hyperpolarising responses to $GABA_A$ receptor activation in many forebrain neurones (36, 37). It also explains why it is not easy to predict whether $GABA_A$ receptor activation will be hyperpolarising or depolarising because Cl^- homeostasis is not static in neurones, even in the adult. Furthermore, E_{GABA} can be near the resting potential membrane potential of a cell thereby making its effects on membrane potential small or even negligible.

A further complication in defining $GABA_A$ receptor activation as being excitatory or inhibitory is that hyperpolarisation and depolarisation may not necessarily be equated with inhibition and excitation, respectively. For excitation to occur (i.e. initiation of action potential firing), a depolarising response must be sufficient to activate the voltage-gated sodium channels that generate the sharp spike of the action potential (38, 39). That is, the membrane must depolarise to the threshold for action potential initiation. A depolarisation in membrane potential that is insufficient to reach threshold for action potential initiation can generate two main responses. First, it can activate other mechanisms such as voltage-dependent channels, which further depolarise the membrane to the threshold for action potential generation (21, 34, 40, 41). Second, it can induce what appears to be a paradoxical inhibition of action potentials through what is called 'depolarising' or shunting inhibition. Whenever the $GABA_A$ receptor channel is open, there is a transient increase in membrane conductance through the pores and thus a decrease in membrane resistance (also called input resistance, R_{in}). During this transient reduction in input resistance, a greater amount of current is required to produce a unit change in membrane potential (from Ohm's law $\Delta V_{membrane} = IR_{in}$). As a result, a depolarisation in membrane potential subsequent to $GABA_A$ receptor activation may render a cell less responsive to other inputs, leading to shunting inhibition (42). In cells with slow membrane time constants (typically big cells with extensive dendritic trees), the resultant slow depolarisation can also inactivate sodium channels needed for action potential generation, causing transient inhibition through this mechanism (43).

The classic response to $GABA_A$ receptor activation in adults is a hyperpolarisation in membrane potential that is typically inhibitory. It is possible, however, for hyperpolarisation to remove inactivation from sodium channels. This can result in rebound action potentials after decay of the inhibitory postsynaptic potential (44–46). There are thus many factors that must be taken into account before classifying a response to $GABA_A$ receptor activation as excitatory or inhibitory.

An important final consideration when examining the effects of $GABA_A$ receptor activation on membrane potential is that of experimental approach (Fig. 3). As noted above, intracellular Cl^- levels are absolutely critical in determining E_{GABA} and so it is essential that the recording configuration does not alter the internal chloride *milieu* of the recorded cell. There two practical ways to achieve this; the first is to use an 'on-cell' recording approach in which an electrode is placed on the cell without any attempt to break the membrane of the cell (Fig. 3A). This allows the investigator to record the firing pattern of the cell (47) without any perturbation of the intracellular environment, or to estimate membrane potential (48). The second approach is to use the 'perforated-patch' approach (Fig. 3B).

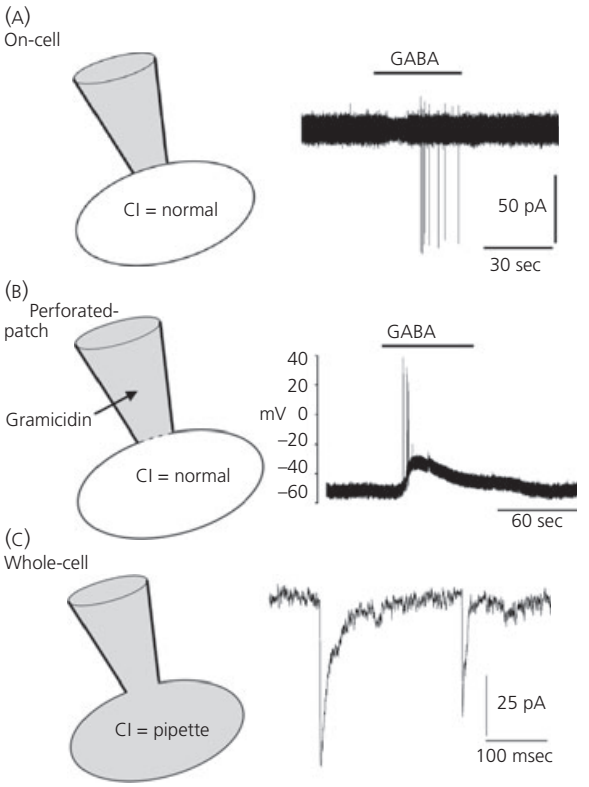

Fig. 3. Schematic diagram showing the different electrophysiological recording modes used to examine $GABA_A$ receptor functioning in gonadotrophin-releasing hormone (GnRH) neurones. (A) On-cell configuration in which the recording electrode (grey) is placed on the cell membrane, a tight seal is formed and currents recorded from the GnRH neurone. The current underlying an action potential is recorded as an action current (in pA); this enables the firing pattern of a GnRH neurone to be monitored without altering the intracellular constituents (e.g. the chloride ion concentration) of the GnRH neurone. The example trace shows a GnRH neurone activated to fire action currents by GABA added to the bathing medium. (B) Perforated patch recording mode in which the presence of an antibiotic (in this case gramicidin) in the patch electrode results in ion-selective pores being made in the membrane beneath the electrode. Gramicidin allows some ions (potassium and sodium) to pass through the pores but not chloride. This enables the membrane potential to be monitored at the same time as maintaining the normal chloride ion concentration of the cell. The example shows a GnRH neurone with a resting membrane potential of approximately −55 mV that responds to GABA application with a short burst of action potentials and more prolonged depolarisation. (C) Whole-cell recording mode in which the membrane within the electrode is ruptured so that the intracellular contents of the cell become dialysed with that of the pipette. In this way, the intracellular ion environment of the GnRH neurone can be manipulated by the contents of the pipette. This mode is commonly used to help isolate or augment a particular current for measurement. In this example, the recording was optimised to monitor Cl^- currents to measure the frequency of activation of $GABA_A$ receptors. The recording on the right shows postsynaptic currents as a result of chloride flow through $GABA_A$ receptors of the recorded cell that were opened by synaptic release of GABA. Note the different timescale of this recording compared to that in (A) and (B). It is important to note that one cannot use this approach to study the physiological response of the recorded cell to activation of those receptors as the chloride ion concentration in the cell is artificial (Kiho Lee and Stephanie Constantin are thanked for their help with preparing this figure).

In this case, an electrode with the antibiotic gramacidin in its tip is placed on the cell membrane. With time, gramicidin forms small pores in the membrane beneath the electrode through which monovalent ions such as sodium and potassium can pass, although these pores are not permeable to chloride ions. Hence, in this configuration, the investigator has a relatively good electrical connection with the cell and can measure firing rate and membrane potential changes without altering the internal chloride ion balance (49). It is important to note here that the most common electrophysiological technique called 'whole-cell' recording (Fig. 3c) is not appropriate for determining the effect of $GABA_A$ receptor activation on membrane potential or firing rate because this method dialyses the intracellular *milieu* of the cell with the solution in the patch electrode buffer, and thus alters the native intracellular [Cl^-]. The whole-cell mode can, however, be used to good effect to examine the dynamics of the individual $GABA_A$ receptors because, by filling the cell with a high Cl^- concentration through the pipette, one can amplify the Cl^- ion movements and observe $GABA_A$ receptor ion channel openings and closings with ease.

GABA inputs, $GABA_A$ receptors and GnRH neurones in the mouse

GnRH neurones receive GABAergic input

One of the most consistent observations revealed during the initial electrophysiological investigations of GnRH neurones in the brain slice preparation was that these cells receive GABAergic input (18, 50). Almost all GnRH neurones exhibit $GABA_A$ receptor-mediated postsynaptic currents (p.s.c.s.) and respond to exogenous GABA (16, 18, 50, 51). In both voltage- and current-clamp recording configurations, the great majority of fast postsynaptic activity at the GnRH neurone soma is attributable to $GABA_A$ receptor activation (18, 50). The remaining fast p.s.c.s. result from the activation of glutamate receptors, principally the AMPA receptor (16, 52–54); however, it is important to note that the glutamatergic component is small compared to the GABAergic component. In experiments in which GABA and glutamate p.s.c.s. have been recorded from GnRH neurones, the rate of $GABA_A$-mediated events is typically five- to ten-fold greater than glutamate events (52, 55–58). The potential role of glutamatergic and GABAergic transmission to distal dendrites remains to be determined (59, 60).

$GABA_A$ receptor signalling occurs in two forms: 'phasic' and 'tonic'. Phasic refers to the normal fast activation of $GABA_A$ receptors within the synapse, whereas tonic represents the activation of extrasynaptic $GABA_A$ receptors by GABA in the extracellular space (61) (Fig. 1). Phasic activation is observed electrophysiologically as the brief opening and closing of $GABA_A$ receptors, resulting in p.s.c.s. Tonic currents are evident as a persistent current that has a sustained influence on membrane potential. Although phasic $GABA_A$ receptor signalling has long been recognised in GnRH neurones, it is only recently that tonic $GABA_A$ signalling has been identified in these neurones (62, 63). Mediated by δ subunit-containing $GABA_A$ receptors and partly dependent upon the activity of glial and neuronal GABA transporters, which clear GABA from the extracellular space, tonic $GABA_A$ receptor signalling was found to hyperpolarise the membrane by approximately 5 mV in mouse GnRH neurones (63). As such, modulation of this tonic GABA current could have important roles in determining the excitability of GnRH neurones.

A feature of phasic $GABA_A$ receptor activity in GnRH neurones in the brain slice preparation is that it often does not appear dependent to any large extent upon electrical activity in the presynaptic GABA terminal. The frequency of $GABA_A$-mediated miniature (action potential independent) p.s.c.s. is often similar to that of spontaneous (action potential dependent and independent) p.s.c.s. (57, 64, 65). This type of activity-independent GABA release is observed in other neurones (66–68); however, its regulation and function remain poorly understood (69, 70). Indeed, a variety of chronic and acute gonadal steroid treatments known to modulate the frequency of $GABA_A$ p.s.c.s. in GnRH neurones (see below) do so by changing the rate of action potential-independent GABA release with no or little measured effect on activity-dependent GABA release (57, 62, 64, 65, 71). Because there is evidence to suggest that activity-independent GABA release is related to the degree of GABAergic input existing at a cell (68), it is possible that the changes in $GABA_A$ miniature postsynaptic currents (m.p.s.c.) frequency represent alterations in the number or extent of GABAergic inputs to GnRH neurones. Unfortunately, little is presently known about GABA-GnRH neurone ultrastructural rearrangements with steroid treatment. On the basis of *in vivo* microdialysis and neurochemical studies it has been suggested that the oestrogen regulation of GABA release in the preoptic area results from a reorganisation of GABA presynaptic terminal dynamics (72). Recent work also suggests retrograde endocannbinoid signalling from GnRH neurones can alter m.p.s.c. frequency, suggesting that signalling at the presynaptic terminal can bring about functional changes that are not dependent on structural rearrangements (73).

It is important to note that all of the $GABA_A$ p.s.c.recordings have been undertaken in the acute brain slice preparation in which many, or possibly most, of the GABAergic (and other) afferents to GnRH neurones have been severed. As such, it may be that much of the activity-dependent GABA input is absent in the slice preparation. Indeed, recordings from other neuronal cell types *in vivo* have revealed that levels of synaptic activity and firing rates are up to 50-fold higher *in vivo* compared with the acute brain slice preparation (74, 75). Establishing the actual rate and pattern of $GABA_A$ p.s.c.s in GnRH neurones in a more intact preparation is an important future goal for this field.

At present, the locations of the GABAergic cell bodies innervating GnRH neurones are not well established. The anteroventral periventricular nucleus (AVPV), a region that has been suspected as providing GABAergic inputs for many years (76–79), has just recently been confirmed using an electrophysiological approach (9). By cutting horizontal brain slices, it was found possible to maintain the AVPV input to GnRH neurones in GnRH-GFP transgenic mice and this was shown to provide a very substantial GABAergic input to GnRH neurones. Another study has examined the potential locations of GABA neurones contributing to p.s.c.s. in GnRH neurones at the time of oestradiol positive feedback by looking for differences between lateral and medial (containing AVPV) sagittal and coronal brain slices

(57). However, with one exception, no differences were detected in $GABA_A$ p.s.c. frequency between any of the slice configurations, or any tetrodotoxin (TTX)-sensitivity found. The exception was that the p.s.c. frequency of GnRH neurones recorded in medial sagittal slices, and exhibiting high p.s.c. frequency at the time of the GnRH surge, was reduced by approximately 50% by TTX or by a cut through the slice made just caudal to the AVPV. One interpretation of this finding is that an activity-dependent GABA input originating caudal to the AVPV, perhaps the suprachiasmatic nucleus, is activated at the time of the surge (57). Further work is required to establish the origins of GABAergic inputs to GnRH neurones.

GnRH neurones express a range of $GABA_A$ receptor subtypes

As mentioned above, the $GABA_A$ receptor is a pentamer made up of different combinations of subunits that define its binding affinity to a variety of allosteric modulators (10). Several methodologies including dual-label *in situ* hybridisation and single cell reverse transcriptase-polymerase chain reaction have been used to define the $GABA_A$ receptor subunits expressed by GnRH neurones in the rodent (50, 62, 63, 80–85). These studies suggest that there are sex differences, as well as postnatal developmental alterations in the α, β and γ $GABA_A$ receptor subunits expressed by GnRH neurones. For example, in the adult female mouse, there is a predominance of α1, α3, α5, β1 and γ2 mRNA in contrast to the male in which transcripts for almost all of the α, β and γ $GABA_A$ receptor subunits can be detected (84). Also, whereas many different subunits are detected in prepubertal GnRH neurones, this appears to become more restricted in adults (50, 81). Much less attention has been paid to the uncommon $GABA_A$ receptor subunits, although the δ and ε subunits have now been identified in these cells (62, 63, 82). Although studies have shown that both androgen and progesterone derivatives are potent allosteric modulators of $GABA_A$ receptors expressed by GnRH neurones (86, 87), the full functional relevance of the many different $GABA_A$ receptors likely to be expressed by GnRH neurones is not known.

The precise locations of $GABA_A$ receptors on GnRH neurones also remain unexplored. Recent studies have shown that GnRH neurones in the mouse extend long dendrites that are often well over 1000 μm in length (88) and often intertwine (89). This, along with evidence that action potentials can be initiated in the dendrites of GnRH neurones (90), has required a re-evaluation of how GnRH neurones receive and integrate afferent input. Although not yet demonstrated, it appears likely that the full length of GnRH neurone dendrite will receive GABAergic inputs. Studies to date show the density of vesicular GABA transporter-containing appositions on the proximal dendrites of GnRH neurones to be double that found on the soma (91). Understanding the location of $GABA_A$ receptors and their spatial relationship to other receptors, particularly glutamatergic receptors (21), will be essential in defining GABA action on GnRH neurones. For example, it has recently been proposed that shared GABAergic synapses on intertwined magnocellular neurone dendrites play a role in synchronising oxytocin neurone activity (92).

Effects of $GABA_A$ receptor activation on immature GnRH neurones

It is now well established that GABA exerts a depolarising and excitatory influence upon many neurones in the embryonic and perinatal brain in altricial species (93). Recordings from immature GnRH neurones indicate that they are probably not an exception to the rule. Experiments using the immortalised GT1-7 GnRH neuronal cell line indicate a depolarising effect of $GABA_A$ receptor activation as GABA induced action potential firing in GT1-7 cells recorded in the 'on-cell' configuration (94). Calcium imaging further revealed that GABA induced increases in intracellular calcium in GT1-7 cells that were dependent upon action potential generation and cell membrane calcium channels. This was the first indication that activation of $GABA_A$ receptors in GnRH neurones might be other than inhibitory, and was followed quickly by a study in which $GABA_A$ receptor activation in these GT1-1 and GT1-7 cells was shown to increase GnRH release (95, 96). The pure nature of GT1 cultures assists interpretation of these studies as secondary effects as a result of other cell types are precluded. It is arguable, however, that the excitatory response of GT1 cells might be the result of an artefact of transformation of the cells, which might result in behaviours different from native GnRH neurones. It is also possible that the co-transporter profile was 'fixed' at a relatively 'immature' stage by an earlier transformation event despite the derivation of this cell line from an adult mouse.

A depolarising influence of $GABA_A$ receptor activation in immature GnRH neurones also receives support from calcium imaging studies undertaken in the embryonic nasal explant model (97, 98). Mouse embryonic GnRH neurones have been shown to exhibit a sharp increase in intracellular calcium levels in response to $GABA_A$ receptor activation; an effect compatible with membrane depolarisation (99).

One study using perforated-patch recordings examined the effects of direct $GABA_A$ receptor activation on prepubertal GnRH neurones (postnatal day 10–30) in the mouse brain slice and found consistent depolarising actions (100). Together, these findings indicate that $GABA_A$ receptor activation exerts a predominant depolarising and excitatory response from embryonic through to pre-pubertal age GnRH neurones.

Effects of $GABA_A$ receptor activation on adult GnRH neurones

Unlike the coherent picture of a depolarising response to GABA in pre-pubertal GnRH neurones, the consequence of $GABA_A$ receptor activation in adult GnRH neurones has been controversial, although a more unified view is beginning to emerge.

The first two studies

The development of GnRH-promoter driven reporter genes allowed the question of GABA action to be addressed in acutely-prepared brain slices from mice (16, 101, 102). Han *et al.* used GnRH neurones expressing beta-galactosidase (GnRH-LacZ), which can convert

substrates to a fluorescent state allowing identification of cells expressing this enzyme. In this model, gramicidin-perforated patch recordings suggested a developmental change in the direction of response to GABA from depolarising to hyperpolarising that resembles that in pyramidal neurones (100). GABA was depolarising in young (day 10–17 postnatal) GnRH neurones but hyperpolarising in cells from more mature mice (day 36–55 postnatal). This suggested a change in response during the peripubertal period and a possible link to puberty onset. However, no such change in response was observed by DeFazio *et al.* (103) using enhanced green fluorescent protein (eGFP)-identified GnRH neurones. Rather, both on-cell and gramicidin-perforated patch recordings of GnRH neurones revealed an excitatory response to $GABA_A$ receptor activation regardless of sex, time of day or age (note that alterations in GnRH neurone response to GABA possibly contributing to reproductive senescence have not been studied by either laboratory).

How to account for these different results? One possibility is that of reporter gene (GFP fluorescence versus LacZ). However, this appears unlikely because the same adult hyperpolarising effects of $GABA_A$ receptor activation have been observed using GnRH-eGFP mouse lines (63, 104); it is perhaps noteworthy that the GnRH-GFP mice used by the two laboratories are different although GFP itself does not appear to confound the data (103). A further difference was that DeFazio *et al.* (103) examined $GABA_A$ receptor activation in the presence of ionotropic glutamatergic receptor antagonists and the study by Han *et al.* (100) did not. This should not alter the direction of response to GABA, particularly given the relative paucity of glutamatergic inputs to GnRH neurones, but becomes important in later studies discussed below.

A main difference between these two studies was duration and type of GABA application. In the study by Han *et al.*, GABA (10–100 μM) was bath-applied for approximately 30 s to 1 min (100); in the study by DeFazio *et al.* (103), GABA (1 mM) was applied briefly and locally for a couple milliseconds [GABA concentrations in the synapse are estimated to be in the low millimolar range (105, 106)]. Bath application of the $GABA_A$ receptor agonist muscimol for 1–3 min invariably generated an initial excitatory response of a barrage of action potentials from GnRH neurones (103). This was followed by a failure to respond to exogenous GABA with either a change in firing rate or membrane potential, and GnRH neuronal quiescence that persisted for several minutes. This quiescence was accompanied by a marked reduction in the membrane (input) resistance of GnRH neurones, typically near 1 GΩ, to approximately 100 MΩ during and for a period after muscimol treatment. Together, these data suggest the suppression of GnRH neurone activity and lack of response to GABA after prolonged activation of the $GABA_A$ receptor could be the result of a combination of pharmacologically-induced collapse of the chloride gradient, shunting inhibition as a result of reduced membrane resistance and/or $GABA_A$ receptor desensitisation. This cannot, in and of itself, account for the membrane hyperpolarisation observed in the study by Han *et al.* in response to GABA (100). Activation of $GABA_B$ receptors would be one possible explanation for this hyperpolarisation; neither study included blockers of $GABA_B$ receptors when examining action potential generation although inclusion of $GABA_B$ antagonists did not alter E_{GABA} (103). However, the hyperpolarisation in the study by Han *et al.* was blocked by a $GABA_A$ receptor antagonist, suggesting activation of $GABA_B$ receptors does not explain these findings (100).

As mentioned above, cation-chloride co-transporters play a major role in setting internal chloride and hence the response to activation of these receptors. Messenger RNA and protein for the chloride accumulating NKCC1 have been demonstrated in murine GnRH neurones (103). Some studies have reported protein for the chloride extruding transporter KCC2 in a subpopulation of GnRH neurones (107), whereas others have not detected this protein (103). In terms of function, the specific NKCC1 inhibitor bumetanide hyperpolarises E_{GABA} in murine GnRH neurones (103) and in terminal nerve GnRH neurones of teleosts (108). This action of bumetanide provides functional evidence for chloride accumulation via NKCC1 at least within GnRH neuronal cell bodies. Thus, at present, data on chloride co-transporters in GnRH neurones at the expression level are somewhat mixed and not especially helpful in resolving the controversy; however, functional data indicate GnRH neurones in brain slices actively accumulate chloride, consistent with a depolarising/excitatory response to $GABA_A$ receptor activation.

The second two studies

The first studies performed in our laboratories used exogenous GABA to evaluate the impact of $GABA_A$ receptor activation on GnRH neurone excitability. This is a valid approach, used widely by electrophysiologists, although it does not answer the important question of how GnRH neurones respond to endogenous GABA. Ideally, to examine this question, one would activate GABAergic afferents and monitor the response of GnRH neurones using one of the approaches that does not alter intracellular chloride milieu. This approach is complicated by our relative lack of understanding of the location of GABAergic neurones that are afferent to GnRH neurones. An alternative approach is to block endogenous signalling via the $GABA_A$ receptor using a specific antagonist and observe the response of GnRH neurones. This is a rather poor surrogate for the following reasons. Bath application of $GABA_A$ receptor blockers effectively treats the entire GnRH neurone, which local application would likely not achieve given the extensive dendrites in some of these cells (89). However, with bath application, all cells in a brain slice are affected, including non-GnRH neurones presynaptic to the recorded neurones. This effectively removes GABAergic signalling via the A-type receptor, which is inhibitory to many hypothalamic neurones in the same brain slice (109). In the cortex and hippocampus, this approach is used as an *in vitro* model of epilepsy because such treatment can cause widespread 'disinhibition' of many neurones in the slice, including excitatory inputs to one's cell of interest (110–112).

Using this approach with a gramicidin perforated-patch and without blocking ionotropic glutamate receptors, studies in the Herbison laboratory (104) found that approximately 70% of GnRH neurones were depolarised by the $GABA_A$ receptor antagonist bicuculline, 20% showed no response and 10% were hyperpolarised. Using extracellular recordings after pretreatment with ionotropic glutamate receptor blockers, experiments in the Moenter laboratory (113), found that

$GABA_A$ receptor antagonists reduced the firing rate of 80% of active GnRH neurones, had no effect on 10% and increased firing in 10%. No increase in firing was observed in quiescent GnRH neurones, indicating quiescence was not a result of $GABA_A$ receptor activation. When the response of GnRH neurones to $GABA_A$ blockers was tested in the absence of the glutamate receptor antagonists, 100% showed an increase in firing rate (113). These results were interpreted in two ways. One interpretation was that this demonstrated the key importance of on-going glutamatergic signalling to the direction of $GABA_A$ receptor responses in GnRH neurones as found elsewhere in the brain (21). The other interpretation was that the removal of $GABA_A$ receptor actions throughout the brain slice resulted in the disinhibition of excitatory inputs to GnRH neurones, again a plausible explanation given work in other brain areas (110–112).

More recent studies

It is important that many different experimental angles and models are used to address difficult questions in a field. A major contribution was made when studies in the Kato laboratory (85) reported that $GABA_A$ receptor activation excited GnRH neurones cultured from a rat GnRH-eGFP model. Using perforated-patch electrophysiology, it was found that GABA exerted a dose-dependent, $GABA_A$ receptor-mediated depolarising action on all rat GnRH neurones tested. A caveat of this study is that the membrane preserved in these adult neurones in short-term culture is primarily perisomatic. Other studies from this group using the same model have shown that $GABA_A$ receptor activation also increases intracellular calcium levels in GnRH neurone somata (114), supporting their electrophysiological findings.

A recent study from the Herbison laboratory (115) has, to their surprise, identified predominant stimulatory effects of $GABA_A$ receptor activation upon intracellular calcium levels in GnRH neurones obtained from the GnRH-pericam transgenic mouse line. This experimental model enables the real-time measurement of intracellular calcium levels in GnRH neurones without any manipulation of the cell in its native environment within the acute brain slice preparation (116). $GABA_A$ receptor activation elevated calcium levels in approximately 70% of prepubertal as well as adult GnRH neurones. This effect involved activation of L-type calcium channels and suggested that $GABA_A$ receptor-mediated depolarisation of GnRH neurones was sufficient to activate these channels.

Another new study from the Herbison laboratory (9) agrees with these more recent results. This study used an angled horizontal brain slice preparation to examine the effects of endogenous GABA inputs originating from the AVPV on GnRH neurones. That investigation demonstrated that low frequency (< 1 Hz) electrical stimulation of the AVPV could evoke monosynaptic responses from GnRH neurones that were mediated predominantly by $GABA_A$ receptor activation. Of the GnRH neurones that were activated by AVPV stimulation, approximately 60% of GABAergic responses were excitatory, approximately 25% neutral and approximately 15% inhibitory to GnRH neurone activity. Thus, it is apparent that endogenous GABA inputs to GnRH neurones can exert both excitation and inhibition, but that excitation predominates.

Together, these more recent studies support the original proposal by DeFazio *et al.* that GABA can provide an excitatory input to adult GnRH neurones (103). Although the majority of GnRH neurones in coronal, sagittal and angled horizontal slice preparations are excited by $GABA_A$ receptor activation, others exhibit the more classical inhibitory response. The subcellular and physiological reasons for this heterogeneity remain to be established. It is interesting to note that researchers examining the GABA responses of hypothalamic suprachiasmatic nucleus neurones have long been involved in a similar controversy as to whether $GABA_A$ receptor activation is excitatory or inhibitory with current perspective being that both responses exist (117–120). One source of variability among studies and among individual cells is that chloride ion transporter activity can be labile. Changes in transporter activity could alter a cell's response to $GABA_A$ receptor activation. In several cases, acute activity-dependent changes in KCC2 function have been found to switch the GABA responses of neurones (24, 121–124). Furthermore, in the context of studies that have been undertaken in culture or in the acute brain slice preparation, it is important to note that cellular damage can rapidly modulate KCC2 activity promoting depolarising effects of $GABA_A$ receptor activation (124–126). Of note, as mentioned above, only a subpopulation of GnRH neurones have been reported to express KCC2 protein (103, 107). It is also important to point out that cellular damage results in reduced membrane resistance and other changes that can alter the ability of a cell to respond to inputs and intrinsic changes that are independent of chloride co-transporters; cellular damage in slices with poor health also impairs the ability to perform the high-quality recordings needed to test these responses.

Modulation of GABAergic transmission to GnRH neurones

Further evidence that can inform this debate is the study of how GABAergic transmission to GnRH neurones varies with different reproductive states. The frequency of GABAergic p.s.c.s. reveals how often afferent GABAergic neurones are signalling to the GnRH neurone being recorded. The amplitude of p.s.c.s. is attributable to both presynaptic changes, such as how much transmitter is released, and postsynaptic changes, such as number and type of receptors and any post-translational modifications that alter conductance through the pore or open probability.

Effects of steroid milieu, nutritional status and neuromodulators on GABAergic transmission to GnRH neurones in sum indicate that those conditions that favour increased GnRH release (mild hyperandrogenemia, oestradiol positive feedback, kisspeptin) increase GABAergic transmission to GnRH neurones and p.s.c. amplitude (55, 57, 64). By contrast, conditions that reduce GnRH release (progesterone, fasting, oestradiol negative feedback) reduce GABAergic transmission and p.s.c. amplitude (18, 57, 64, 65). The sole exception to date to a direct relationship between the frequency of GABAergic transmission and that of GnRH activity is a study of the effects of long-term treatment with supraphysiological levels of synthetic androgens to mimic anabolic steroid abuse, (62). In that study, an inverse relationship between these parameters was observed. An

interesting topic for future study will be to determine whether androgen abuse alters chloride co-transporter function in GnRH and other neurones.

It is interesting that amplitude and frequency of GABAergic p.s.c.s. in GnRH neurones tend to move in the same direction. The smaller p.s.c.s. detected in GnRH neurones with reduced activity would be more likely to generate subthreshold depolarisations rather than depolarisations that result in spike generation. This could compound any effect of reduced frequency of GABAergic drive. Reduced temporal summation of low frequency GABAergic p.s.c.s. would also reduce the occurance of GABA-driven depolarisation that is sufficiently large to reach action potential threshold. Although data from studies such as these provides circumstantial evidence rather than direct evidence of the consequence of $GABA_A$ receptor activation, they are largely consistent with an excitatory role.

Summary and perspectives

Over approximately the past decade, the thinking on the consequence of $GABA_A$ receptor activation on GnRH neurones has evolved from a fairly stark controversy to a consensus. It appears that considering GABA as purely inhibitory or excitatory is to some extent an oversimplification as both responses can be observed. Furthermore, the integration of GABAergic with other ionotropic and neuromodulatory inputs (59, 60, 127–130) and intrinsic conductances will sculpt the ultimate membrane response. For example, on-going glutamatergic activity in the vicinity of the $GABA_A$ receptor synapse may influence the $GABA_A$ receptor response. Equally, it is possible that $GABA_A$ receptor activation at the cell body could have different net effects on GnRH neurone activity compared to activation of $GABA_A$ receptors located on the dendrites (131).

GnRH neurones are regulated by a number of influences and the importance of continued function of this system for passing on the genome of a species suggests multiple regulatory systems, and also that redundant systems will operate in the absence of specific signalling. In this regard, a recent study demonstrated continued fertility in a mouse model with knockdown of $GABA_A$ receptor-mediated signalling in GnRH neurones via germline deletion of the γ-2 subunit (132). Similarly, there is GnRH neuronal activity even in the absence of the most potent stimulator identified to date in these neurones: kisspeptin (58, 133). Together, these observations point to a need to move away from all-or-none statements to more integrated viewpoints when considering the impact of specific neurotransmitters on GnRH neurones. They also point to the importance of cellular level observations in examining mechanisms. In this regard, the relationship between the predominantly excitatory actions of $GABA_A$ receptor activation on GnRH neurones and the primarily suppressive effects of GABA on LH secretion *in vivo* (2) are most likely explained by indirect influences of even narrowly focused drug treatments *in vivo*.

The present status of the GABA story, with mainly excitatory but also inhibitory responses to this transmitter, suggests several questions that need to be addressed in future studies. Is GnRH neurone intracellular chloride differentially regulated in various subcellular compartments (dendrites, soma, terminals)? Are chloride co-transporters modulated by reproductive state, neural inputs or on-going patterns of electrical activity? What happens with ageing? Does GABA potentially have a role in synchronising GnRH neurones as it does in some hippocampal networks (134)? The answers to these and other questions will further evolve our understanding of the role of GABA in sculpting GnRH neuronal activity. And finally, how might our present view of $GABA_A$-receptor mediated signalling in GnRH neurones be revised when *in vivo* recordings of this response become possible?

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