ELSEVIER

Molecular and Cellular Endocrinology 185 (2001) 185–194

MCE
Molecular and Cellular Endocrinology

www.elsevier.com/locate/mce

Molecular and cellular properties of GnRH neurons revealed through transgenics in the mouse

Allan E. Herbison *, Jean-Remi Pape, Sharon X. Simonian, Michael J. Skynner, Joan A. Sim

Laboratory of Neuroendocrinology, The Babraham Institute, Cambridge CB2 4AT, UK

Abstract

Recent advances in the use of gonadotropin-releasing hormone (GnRH) promoter-driven transgenics in the mouse are beginning to open up the once elusive GnRH neuronal phenotype to detailed molecular and cellular investigation. This review highlights progress in the development of GnRH promoter transgenic constructs and the understanding of murine gene sequences required for the correct temporal and spatial targeting of transgenes to the GnRH phenotype in vivo. Strategies enabling the identification of single, living GnRH neurons in the acute brain slice preparation are allowing gene profiling and electrophysiological experiments to be undertaken. Results so far indicate that, like other neurons, GnRH cells express a variety of sodium, potassium and calcium channels as well as GABAergic and glutamatergic receptors which are responsible for determining the membrane properties and firing characteristics of the GnRH neuron. Many of these receptors and channels appear to be expressed heterogeneously within the GnRH phenotype. Furthermore, several display distinct postnatal developmental expression profiles which are likely to be of consequence to the development of synchronized, pulsatile GnRH secretion in the adult animal.

Keywords: Calcium; Electrophysiology; GABA; Glutamate; GnRH; LHRH; Potassium; Transgenic

1. Introduction

The release of gonadotropin-releasing hormone (GnRH) from nerve terminals located in the median eminence of the hypothalamus is the principal regulator of pituitary gonadotroph functioning and, thus, fertility in all mammalian species. The decapeptide GnRH was discovered in the early 1970s (Amoss et al., 1971; Schally et al., 1971) and the first immunocytochemical mappings of neurons expressing the decapeptide undertaken shortly thereafter (Barry et al., 1973). Unusually, the GnRH neuron cell bodies were not found to exist within any well defined nucleus of the brain but, instead, were scattered throughout the basal forebrain as a 'continuum' of neurons stretching from the olfactory lobes to the basal hypothalamus (Silverman et al., 1994). This peculiar distribution seems most likely to arise from the extraordinary migration of the GnRH neurons from the olfactory placode into the brain during embryogenesis (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). While the relevance of this unusual origin to the functioning of GnRH neurons in the adult is unclear, the resulting scattered topography of the GnRH neurons has created many problems in the investigation of this phenotype in the postnatal brain. Not only is the selective modulation of GnRH neurons in vivo impossible using conventional techniques, but the inability to gain access to living neurons has prohibited any detailed molecular or cellular characterization. As such, we know very little about the single most important neuronal phenotype regulating fertility.

2. Strategies for evaluating GnRH neurons

In the last decade there have been numerous attempts to circumvent the experimental difficulties imposed by the scattered distribution of the GnRH neurons. One has been that of using multi-unit recording electrodes

* Corresponding author. Tel.: +44-1223-496-528; fax: +44-1223-496-022.

E-mail address: allan.herbison@bbsrc.ac.uk (A.E. Herbison).

0303-7207/01/$ - see front matter
PII: S0303-7207(01)00618-9

positioned in the region of the median eminence in an attempt to provide an index of massed, or individual, GnRH neuronal activity (Wilson et al., 1984; Cardenas et al., 1993). This strategy has been successful in providing a near perfect correlation between multi-unit activity in the basal hypothalamus and pulsatile LH secretion in several mammalian species (Kawakami et al., 1982; Wilson et al., 1984; Mori et al., 1991). However, the interpretation of changes in multi-unit activity which accompany steroid manipulation, and particularly its absence at the time of the LH surge, have been hampered by an incomplete understanding of the nature and origins of the multi-unit recordings.

Another approach to the investigation of the GnRH phenotype has been that of generating immortalized GnRH-expressing cell lines (Mellon et al., 1990; Radovick et al., 1991) and, arguably, this strategy has had the single biggest impact upon GnRH neurobiology. Both Mellon (1990) and Radovick (1991) and co-workers achieved targeted oncogenesis of the GnRH phenotype using promoter transgenics in which 1–2 kb of rat and human GnRH promoter was linked to the SV40 T antigen oncogene. The various GnRH cell lines provided the first simple experimental model for the investigation of GnRH-expressing neurons and are now used extensively in the analysis of GnRH gene expression, peptide processing, secretion and GnRH neuron electrical activity (Weiner et al., 1992; Krsmanovic et al., 1996; Wierman et al., 1996; Zhen et al., 1997; Nelson et al., 1998). Although these cell lines have undoubtedly represented a very useful tool for those interested in GnRH neurons, their relationship to native GnRH neurons in vivo has always been controversial (Selmanoff, 1997). In addition to concerns over the effects of tumorigenesis on these cells, it is not clear what developmental stage they might each represent and how their isolation from glial and other neuronal phenotypes may effect their properties. While it seems reasonable to believe that these cells lines are useful models for the investigation of GnRH gene regulation (Wierman et al., 1996; Nelson et al., 1998), their relevance to GnRH neuron physiology in vivo is much less clear.

The most recent approach for investigating the GnRH phenotype has involved the use of various GnRH promoter transgenic strategies in the mouse. This has provided the first means through which the molecular and cellular characteristics of the GnRH neurons can be assessed in situ within the acute brain slice preparation. We present here a brief review of recent work in the postnatal mouse model and highlight the initial insights into the molecular and cellular properties of these elusive neurons.

3. How much GnRH sequence do you need to target the GnRH phenotype

The principal concern in the use of promoter-driven transgenics to target neuronal phenotypes is that of achieving a high level of selective reporter expression. In general, the targeting of neuropeptidergic phenotypes with promoter-driven transgenics has proven to be extremely difficult (Waschek, 1995). Whereas, some genes such as growth hormone can be successfully targeted to pituitary cells with as little as 200 bp of promoter sequence (Lira et al., 1988), the appropriate expression of transgenes in specific neuropeptide-expressing phenotypes has often required much larger constructs. For example, even 15 kb of somatostatin gene sequence fails to target somatostatinergic neurons in the mouse brain (Rubinstein et al., 1992). Thus, the first obstacle to overcome in the use of promoter transgenics to evaluate the GnRH phenotype has been that of establishing how much GnRH sequence is required to drive transgene expression in the correct spatially and temporally selective manner.

Remarkably, the first report involving GnRH transgenics was published well over a decade ago and involved the use of 13.5 kb murine GnRH transgene in a gene therapy approach to restore fertility to the hypogonadal (*hpg*) mouse (Mason et al., 1986). The *hpg* mouse has a 33 kb deletion involving the distal half of the GnRH gene, which results in an absence of GnRH peptide and infertility. This strategy resulted in the expression of GnRH peptide within GnRH neurons, as well as in a small number of cells located in the paraventricular nucleus and bed nucleus of the stria terminalis (BNST), and restored normal fertility to both male and female mice. This important study provided the first evidence that 13.5 kb of GnRH sequence including both 5′ and 3′ elements was sufficient to direct expression to the GnRH phenotype in the mouse.

In order to further assess the elements of the murine GnRH gene required to target the GnRH population in the mouse, we examined the expression patterns of a variety of GnRH gene fragments coupled to the lacZ reporter (Skynner et al., 1999; Pape et al., 1999). Following on from the work of Mason and colleagues (1986), we began our studies by using the same murine 13.5 kb GnRH gene fragment in which we positioned the lacZ cassette into exon II sequence coding for GnRH to prevent over-expression of GnRH in transgenic mice. Using X-gal histochemistry and antibodies directed against the lacZ product β-galactosidase (βgal), we have been able to determine the precise cellular expression patterns of the transgene in many separate lines of GnRH–lacZ (GNZ) and GnRH–Nuclear Localizing cassette–lacZ (GNLZ) mice (Skynner et al., 1999; Pape et al., 1999;

Simonian et al., 2000, Fig. 1). We found that approximately 85% of the GnRH population was targeted by this construct and, additionally, that transgene expression followed the correct temporal pattern in migrating GnRH neurons during embryogenesis (Skynner et al., 1999). It remains unclear why approximately 15% of the GnRH phenotype do not appear to express the reporter. However, we do find individual GNLZ mice in which 100% of the GnRH neurons are expressing the transgene (Pape and Herbison, unpublished) and presently believe that all GnRH neurons do in fact express the transgene but that some do so at levels well below our threshold for detection.

In a further series of experiments (Pape et al., 1999) we examined the effects of both 3′ and 5′ deletions of the 13.5 kb GNZ construct upon reporter expression in the GnRH neurons. These studies showed that while deletion of sequence 3′ to exon 2 had no effect upon transgene expression in GnRH neurons, it did result in the presence of substantial 'ectopic' reporter expression throughout the brain. Subsequent deletion of 5′ GnRH promoter sequence from 5.2 to 2.1 kb resulted in a small but significant drop in transgene expression within the GnRH population. Importantly, mice bearing constructs with only 1.7 kb of 5′ sequence showed no transgene expression in the GnRH population (Fig. 2).

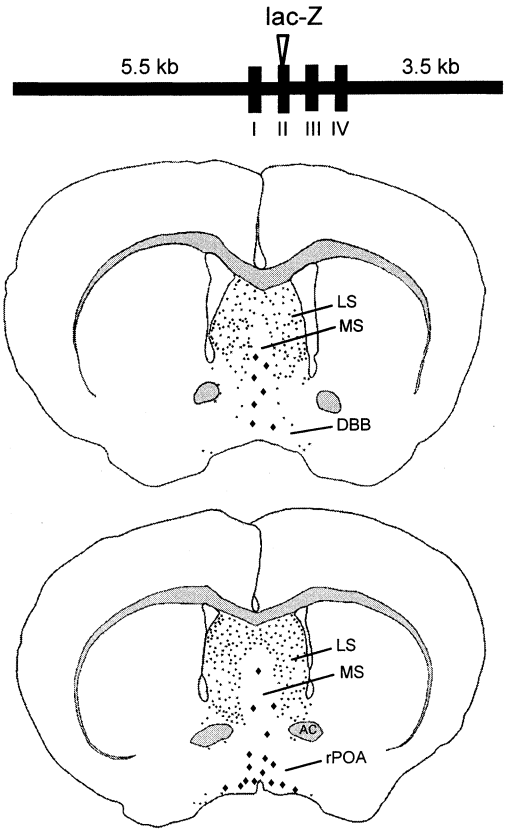

Fig. 1. Spatial patterns of transgene expression in GNZ mice. Top, schematic representation of the 13.5 kb construct used to create the 5.5-GNZ-3.5 mice. The lacZ cassette is inserted in exon II. Bottom, camera-lucida diagrams showing the distribution of transgene-expressing cells at two levels of the mouse forebrain in GNZ mice. Black dots represent βgal-immunoreactive neurons located within several divisions of the lateral spectrum (LS) while diamonds represent the βgal-immunoreactive GnRH neurons. AC, anterior commissure; MS, medial septum; DDB diagonal band of Broca (rPOA, rostral preoptic area).

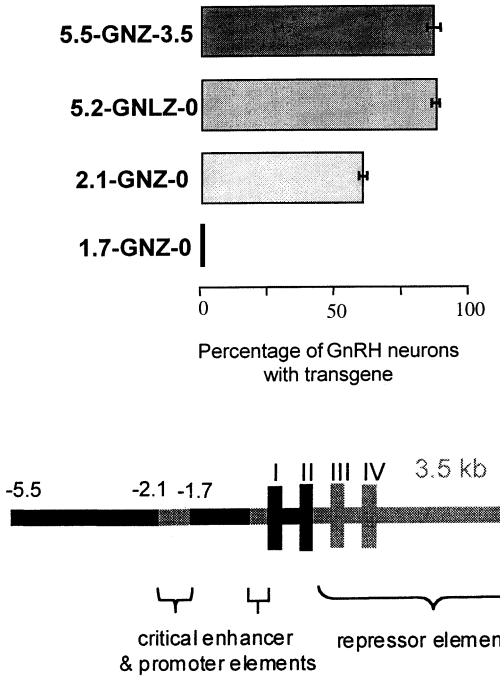

Fig. 2. GnRH gene elements required for GnRH gene expression in vivo. Top, percentage of adult male GnRH neurons with detectable βgal immunoreactivity in transgenic mice bearing GNZ and GnRH nuclear-localizing lacZ (GNLZ) transgenes with different amounts of 5′ and 3′ sequence. For example, 5.5-GNZ-3.5 constructs have 5.5 kb of 5′ and 3.5 kb of 3′ sequence. Reproduced with permission from Pape et al. (1999). Bottom, schematic illustration of identified regions of the murine GnRH gene likely to contain enhancer and GnRH restriction elements regulating gene expression in vivo.

Together, these experiments revealed that the GnRH neuron can indeed be targeted with promoter transgenics and that elements between exon 2 and 3.5 kb of 3′ sequence are important in helping to restrict GnRH gene expression to the GnRH phenotype while an important 400 bp enhancer region exists between −2.1 and −1.7 kb in the GnRH promoter (Fig. 2). It is interesting to note that sequences between −2.4 and −2.0 kb in the mouse are homologous but not exactly identical to characterized enhancer sequence in the rat GnRH gene (Chandran and DeFranco, 1999). Our in vivo findings in the mouse are not, however, the same as those of Wolfe et al. (1996) who used 3.8–0.5 kb of human GnRH sequence to direct expression of the luciferase reporter in transgenic mice. Furthermore, recent studies by Lawson and colleagues (Soc. Neurosci. Abst. 25; 695.1), suggest that as little as a 300 bp of rat minimal enhancer coupled to ∼200 bp of rat

proximal promoter are sufficient to direct transgene expression to GnRH neurons in the mouse. Together, these observations serve to reinforce the idea of marked species differences within the GnRH enhancer and proximal promoter (Kepa et al., 1996; Zakaria et al., 1996).

While these data clearly show that it is possible to target the GnRH phenotype with a promoter transgenic approach, it would appear that quite large amounts of murine 5′ and 3′ GnRH sequence are required to both target these cells accurately and also stop GnRH expression in other cells within the mouse brain.

4. 'Ectopic' transgene and GnRH expression in the mammalian brain

It was evident from our studies, and those of Mason and colleagues (1986), that neurons outside the classical distribution of the GnRH neurons were also expressing the transgene even when 13.5 kb of murine GnRH sequence was employed. Specifically, we found that neuronal populations located in the lateral septum (LS), BNST and lateral olfactory tract were also targeted by the transgene (Fig. 1), albeit at much reduced levels (Skynner et al., 1999). This phenomenon was consistent in all three GNZ lines, subsequent GNLZ lines, and also present in an independent GNZ line (Spergel et al.; Soc. Neurosci. Abst. 24, 237.9). At first this appeared to be an example of the rather typical 'ectopic' transgene expression seen in many neuropeptidergic transgenic lines which is thought to result from the spurious activation of short gene sequences by cells with appropriate transcription factor environments (Waschek, 1995). However, it was also possible that the transgene was reporting on a real phenomenon.

Through the use of an enhanced immunocytochemical procedure we were able to show that authentic GnRH is, in fact, expressed by both lateral septal and BNST transgene-targeted cell populations, in addition to the classic GnRH neurons, during embryogenesis and early postnatal life (Skynner et al., 1999, Fig. 3). GnRH immunoreactivity and transgene expression were found to parallel each other in all three brain regions up until around the first postnatal week when GnRH immunoreactivity in the LS and BNST declined whilst transgene expression persisted (Fig. 3). A further GnRH-expressing population located in the tectum, which had been discovered previously in the mouse brain (Wu et al., 1995), was also detected to contain both transgene and GnRH and, in this case, both proteins were only transiently detected (Fig. 3). At present we believe that lacZ expression in the LS and BNST of adult mice results from the lack of a critical repressor element in the 13.5 kb GnRH sequence which is used in late development to switch off GnRH gene expression in these areas (Fig. 2). How much GnRH sequence might be required to enable this inactivation is unknown but could conceivably require many thousands of bases (Quinn, 1996).

Through use of the Pax-6 null mouse which fails to develop an olfactory placode, we were further able to demonstrate that, unlike the classic GnRH neurons, these novel GnRH-expressing populations did not originate from the olfactory placode (Skynner et al., 1999). In contrast to the LS and BNST, transgene detected in other regions of the brain following deletion of 3′ elements of the GNZ construct (Fig. 2) was not found to be paralleled by the presence of GnRH immunoreactivity at any time point in development and, therefore, is likely to represent true ectopic transgene expression. Although the roles of these new GnRH-expressing neurons in the embryonic LS, BNST and tectum are presently unknown, their discovery through GnRH reporter transgenics provides a good illustration of the unexpected benefits of this type of strategy.

5. Profiling neurotransmitter receptor expression in GnRH neurons

The scattered distribution of the GnRH neurons has made it particularly difficult to determine precisely which molecules are expressed by this phenotype. Until recently the only avenue available to address this issue in native GnRH neurons had been that of dual-labeling immunocytochemistry and/or in situ hybridization. Using GnRH promoter-driven transgenics, two new approaches have become available in the mouse.

The first approach has been that of obtaining the cytoplasmic contents of single, in situ GnRH neurons for multiplex single cell reverse transcription polymerase chain reaction (scRT-PCR). This technique not

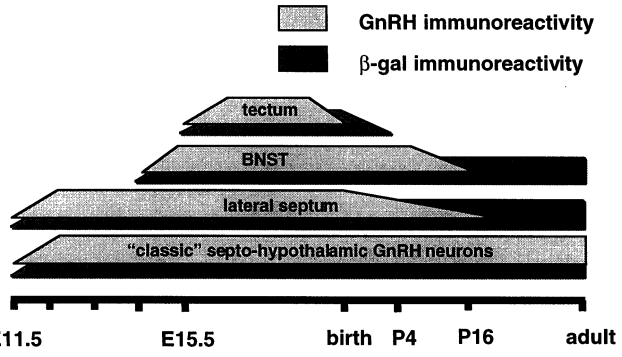

Fig. 3. Schematic diagram indicating the developmental profile of βgal- and GnRH-immunoreactivity in the septo-hypothalamic GnRH neurons, lateral septal, BNST and tectal cell populations in the mouse. E11.5, embryonic day 11.5; P4, postnatal day 4. Reproduced with permission from Skynner et al. (1999).

only enables the existence of any cloned gene to be evaluated but also allows investigation into the presence of multiple transcripts within a single GnRH neuron. This is particularly important for heterooligomeric receptors which display different functional properties depending upon the subunits incorporated into receptor. To this end, we initially concentrated on establishing the profile of $GABA_A$ receptor subunits expressed by postnatal GnRH neurons in the female mouse (Sim et al., 2000). The $GABA_A$ receptor is a heteropentameric receptor of likely 2α, 2β and 1γ subunit stoichiometry, where the different combinations of subunits have marked effects upon the biophysics of the channel as well as its allosteric modulation (Barnard et al., 1998). These studies revealed that a wide range of $GABA_A$ receptor subunit mRNAs were expressed by GnRH neurons in the neonatal and juvenile periods but that this then narrowed to a predominantly α1, α5, β1, γ2 subunit mRNA complement following puberty (Fig. 4). Interestingly, a different $GABA_A$ receptor subunit mRNA profile was detected in GnRH neurons of adult male mice (Pape et al., 2001). Combinatorial analysis of subunit transcript presence in female mice suggested that an $\alpha5\beta_X\gamma2$-type profile was dominant throughout postnatal development and that the presence of other $GABA_A$ receptor isoforms was likely to be down-regulated prior to puberty (Sim et al., 2000). By analyzing GnRH neurons on a spatial basis, we also noted that the down-regulation in subunit heterogeneity was more prominent in GnRH neurons located in the rostral preoptic area compared with those in the medial septum (Sim et al., 2000). This represents the only marked difference we have encountered so far between GnRH neurons located in these two brain regions of the mouse.

The second approach facilitated by GnRH transgenics involves the simplification of immunocytochemical labeling of these neurons. Since the lacZ transgene is targeted to the nucleus of the GnRH neurons in GNLZ mice, this provides a nuclear marker of the GnRH phenotype. This enables cytoplasmic- or membrane-associated antigens to be detected by immunocytochemistry in GnRH neurons without the need to resort to dual immuofluorescence. In the simplest protocol, brain sections from GNLZ mice can be quickly processed by X-gal histochemistry or βgal immunocytochemistry to 'mark' the nuclei of the GnRH neurons before undergoing peroxidase-based immunocytochemistry for the cytoplasmic/membrane antigen of interest. In principal, the same advantage applies to GnRH-GFP mice, where only a single immunofluorescent labeling is now required as the GnRH neurons are now endogenously marked by GFP. We have used the Xgal/βgal protocol to assess the expression patterns of specific $GABA_A$ and NMDA receptor subunit proteins by postnatal

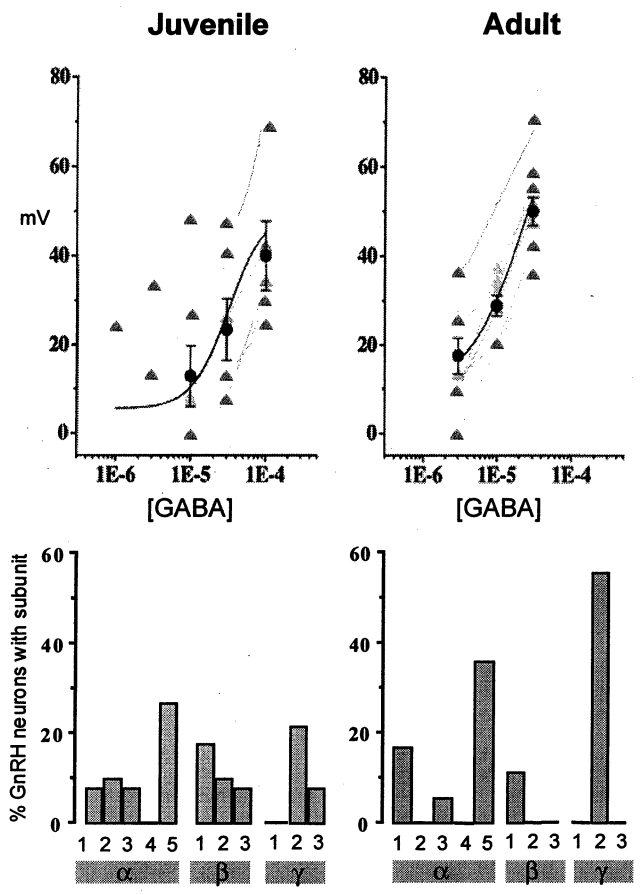

Fig. 4. Increasing homogeneity in $GABA_A$ receptor signaling in GnRH neurons with postnatal development. Top, GABA dose–response relationships of seven juvenile (left) and seven adult (right) preoptic area GnRH neurons in female mice recorded under conditions of isometric chloride ion concentrations (which make GABA responses depolarizing). Graphs show the membrane depolarization responses evoked by 1–100 μM GABA in individual juvenile (linked grey triangles) and adult GnRH neurons, with mean (±S.E.M.) values superimposed as black symbols. Bottom, profile of $GABA_A$ receptor subunit mRNAs detected by scRT-PCR in preoptic area GnRH neurons of juvenile (left) and adult (right) female mice. Vertical bars give the percentage of GnRH neurons found to express each of the α, β and γ subunit transcripts. Reproduced with permission from Sim et al. (2000).

GnRH neurons (Simonian et al., 2000; Simonian and Herbison, 2001). Importantly, the late postnatal up-regulation in $GABA_A$ receptor γ2 subunit mRNA expression observed in scRT-PCR experiments (Fig. 4, Sim et al., 2000) was also seen in γ2 subunit immunoreactivity (Simonian et al., 2000). More strikingly, expression of the obligatory NR1 subunit of the NMDA channel was found to increase 5-fold in GnRH neurons between postnatal days 5 and 15 (Simonian and Herbison, 2001). The results of these studies have indicated clear but distinct patterns of $GABA_A$ and NMDA receptor up-regulation in the majority of GnRH neurons leading up to puberty (Fig. 5).

6. Visualizing GnRH neurons for electrophysiological analysis in the mouse

One of the most important goals of current GnRH transgenic initiatives has been that of enabling living GnRH neurons to be visualized and investigated in situ in the acute brain slice procedure. The first reports of success (Spergel et al., 1999; Suter et al., 2000) have involved the production of transgenic mice bearing constructs comprised of approximately 3.5 kb of murine 5′ GnRH sequence linked to the endogenously fluorescent molecule green fluorescent protein (GFP). Spergel et al. (1999) demonstrated that approximately 65% of the GnRH population was sufficiently fluorescent to be detected in the acute brain slice procedure. Using this fluorescent marker these workers undertook a series of electrophysiological studies demonstrating the presence of $GABA_A$, AMPA and NMDA channels in nucleated-patch recordings taken from postnatal GnRH neurons. A second line of GnRH-GFP mice was made by Suter et al. (2000) and similarly used to undertake whole-cell electrophysiological recordings which showed that, like other neurons, these cells express voltage-activated sodium channels. However, in the case of the latter transgenic mice, made with an enhanced GFP molecule, approximately 95% of the GnRH neurons were reported to be endogenously fluorescent.

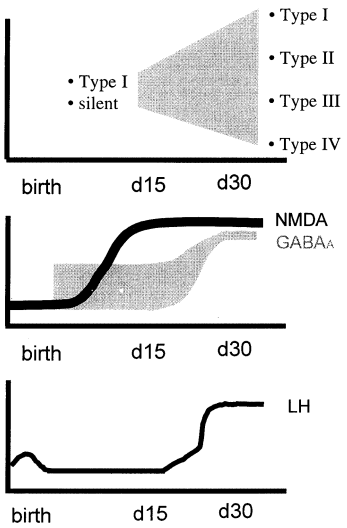

Fig. 5. Schematic diagram indicating the principal changes identified in GnRH neurons over postnatal development in the mouse. Top, based upon the intrinsic membrane properties of GnRH neurons, it appears that only two types are evident in the juvenile mouse, one of which is unable to fire action potentials (silent cells) while at least four types are evident in the mature female. Middle, the expression profiles of NMDA-NR1 and $GABA_A$ receptor subunits in GnRH neurons across puberty. Multiple $GABA_A$ receptor subunits are present in neonatal GnRH neurons and this narrows to an α1, α5, β1, γ2-type subunit complement which also becomes more abundant in adult GnRH neurons. Bottom, the profile of LH, and likely GnRH, secretion in the mouse over development with puberty beginning around day 30.

In light of the presence of transgene-expressing cells in the LS and BNST, as well as the need for 3′ GnRH sequence to prohibit ectopic transgene expression in the region of the GnRH neurons (Pape et al., 1999), it is interesting that 'ectopic' GFP expression has not been reported in either of these transgenic lines. One likely explanation is that the level of GFP expression in lateral septal and BNST, as well as true 'ectopic' cells, is below the threshold for detection with conventional fluorescent microscopes. Certainly, the level of transgene expression in lateral septal cells is known to be at least 5-fold lower than in GnRH neurons (Skynner et al., 1999). Nevertheless, GFP has proved to be a useful fluorescent reporter with which to identify the scattered GnRH neurons. While such GnRH-GFP mice likely represent as big a boost to the field of GnRH neurobiology as the production of the GnRH-expressing cell lines, it is important to remember that the effects of fluorescence illumination and GFP expression on cell physiology are not yet established.

Another approach for identifying living GnRH neurons has been to use fluorescent βgal substrates in conjunction with GNLZ transgenic mice. In this procedure, membrane permeant derivatives of molecules such as fluoroscein-di-β-galactopyranosides (Zhang et al., 1991) enter cells whereupon they are cleaved by βgal to fluorescent moieties. This approach also enables the detection of living GnRH neurons in the slice and can be used to isolate GnRH neurons for electrophysiological, morphological and single cell RT-PCR experiments. However, in the course of developing this methodology, we realized that bipolar GnRH neurons could be identified in the acute brain slice preparation by their neuroanatomical position, bipolar morphology, and orientation without the need for a fluorescent marker (Sim et al., 2000; Sim et al. 2001a). Using post-patching scRT-PCR to identify the presence of GnRH transcripts, we found that around half of such morphologically-identified cells were genuine GnRH neurons. While this approach has its own problems, such as the investigation of exclusively bipolar GnRH neurons, and is substantially more laborious, it does have the great benefit of enabling electrophysiological recordings to be made from unmodified GnRH neurons.

7. Basic membrane properties of GnRH neurons

Whole-cell patch clamp recordings of over 75 unmodified GnRH neurons in juvenile and adult mice have shown that GnRH neurons fire action potentials and exhibit distinguishable voltage–current relationships in response to hyperpolarizing and depolarizing

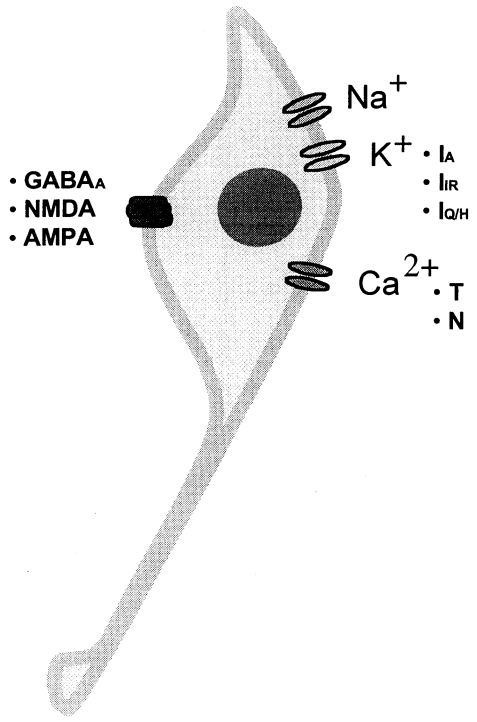

Fig. 6. Summary of ion channels and neurotransmitter receptors known to be expressed by postnatal GnRH neurons in the mouse.

current injections (Sim et al., 2001a). Based upon their patterns of inward rectification, rebound depolarization, and ability to fire repetitively, we have classified GnRH neurons in intact adult females as belonging to one of four distinct cell types (Sim et al., 2001a). The GnRH neurons of juvenile animals exhibited passive membrane properties similar to those found in adults. Interestingly, only two types of GnRH neurons were identified in juveniles, one of which resembled the most prominent type found in adults and the other, comprising about one third of all juvenile GnRH neurons recorded, which did not fire action potentials. Afterdepolarization and afterhyperpolarization (AHP) potentials were observed following single action potentials in sub-populations of each GnRH neuron type. However, AHPs and tetrodotoxin-independent calcium spikes were encountered more frequently in juvenile GnRH neurons compared with the adult suggesting developmental changes in calcium dynamics within GnRH neurons (Sim et al., 2001a).

These recordings of unmodified postnatal GnRH neurons (Sim et al., 2001a), together with those of GFP-tagged GnRH neurons by Spergel (1999) and Suter (2000) and co-workers, have indicated the presence of a variety sodium, potassium (I_{IR}, I_A, $I_{Q/H}$) and calcium channels (N&T-type) in GnRH neurons (Fig. 6). In essence, GnRH neurons appear to express many of the same channels as other neurons and no specific electrophysiological 'fingerprint' has been immediately obvious. However, these recordings have demonstrated that significant heterogeneity exists in the basic membrane properties of GnRH neurons and provided direct evidence for the existence of functional heterogeneity within the GnRH phenotype (Sim et al., 2001). Intriguingly, we have found that this heterogeneity exists in a development-specific manner and that the full repertoire of GnRH heterogeneity is only evident following puberty when these neurons are controlling reproductive functioning (Fig. 5). While such studies are critical in characterizing the GnRH neuron, they have, at present, generated more questions than they have solved. Among other issues, it will now be important to determine the nature of the developmental changes in calcium handling and firing ability in these neurons and the physiological roles of the different types of GnRH neurons in the mature animal.

8. GABAergic regulation of the postnatal GnRH neuron

Earlier studies utilizing the olfactory explant technique had shown that embryonic GnRH neurons expressed functional $GABA_A$ receptors (Kusano et al., 1995) and Spergel et al. (1999) had further demonstrated their presence in postnatal GnRH neurons in the mouse. In a further set of studies we undertook a detailed examination of the electrophysiological actions of GABA upon postnatal GnRH neurons using the whole-cell patch clamp technique (Sim et al., 2000). In these experiments we revealed that all GnRH neurons in juvenile and adult female mice are subjected to a profound GABAergic barrage. Much of this GABA release is action potential independent and, as found elsewhere in the brain (Otis et al., 1991), likely represents the quantal or spontaneous, calcium-independent release of GABA from terminals.

Under circumstances of constant ligand, it became particularly important to assess the properties and possible changes involving the $GABA_A$ receptors expressed by GnRH neurons. Surprisingly, we found marked heterogeneity in the sensitivity of individual GnRH neurons to GABA in juvenile mice; some GnRH neurones displayed high sensitivity to GABA whilst others were relatively insensitive (Fig. 4). Importantly, this marked heterogeneity in response to GABA was not found in GnRH neurons of adult females where all neurons now responded to GABA with similar dose-response relationships in the highly sensitive range (Fig. 4). The changes in the heterogeneity of the GnRH population response to GABA were paralleled by the results of the scRT-PCR analyses where juvenile GnRH neurons were found to express many different $GABA_A$ receptor subunit mRNAs while adults exhibited a more restricted subunit profile (Fig. 4).

While these findings again raise many questions, they also serve to highlight the importance of $GABA_A$ receptor signaling to the GnRH neuron. Together with the subunit profiling data, they demonstrate an unusual pattern of late postnatal reorganization of the $GABA_A$ receptor within the GnRH phenotype. Intriguingly, this pattern of declining $GABA_A$ receptor heterogeneity during late postnatal development is opposite to that of increasing heterogeneity in the expression of functional potassium and calcium channels in these neurons (Fig. 5). The GnRH neuron is a somewhat unusual neuronal cell type in that it only becomes fully functional during late postnatal development and, as such, it is perhaps not surprising to find multiple examples of 'delayed' channel plasticity in these cells.

What might be the consequences of this $GABA_A$ receptor re-organization? If the co-ordination of GnRH neurons to produce pulsatile activity was dependent upon GABAergic interconnections, as seems possible from work in the rat (Herbison et al., 1991), then it might be important for GnRH neuron $GABA_A$ receptors to function in a relatively homogeneous manner. If correct, then the developmental transition to relative homogeneity in the expression and functioning of $GABA_A$ receptors on GnRH neurons could be seen as a prerequisite for these cells to act in a synchronized, pulsatile manner in the adult female. Although we have not yet established the functional link between these changes in GnRH neuron $GABA_A$ signaling and the neural control of fertility, the temporal coincidence of events, associated with evidence for a role of GABA in puberty in the female monkey (Terasawa and Fernandez, 2001) and rodent (Feleder et al., 1996), is compelling.

From another perspective, if gonadal steroid derivatives such as allopregnanolone were to be involved in the physiologically-relevant allosteric modulation of $GABA_A$ receptors on all GnRH neurons, as they are with other hypothalamic phenotypes (Brussaard and Herbison, 2000), the establishment of a degree of $GABA_A$ receptor homogeneity within the adult phenotype would be important. We have recently shown that this is indeed the case as allopregnanolone was found to directly modulate $GABA_A$ receptor functioning in all adult female GnRH neurons (Sim et al., 2001b). Thus, from a variety of angles, it may be important that $GABA_A$ receptor signaling becomes more or less homogeneous within the GnRH population as it transcends into a likely synchronized, episodically active neuronal network in the adult.

9. Conclusions

So far, transgenic methodologies have played a very considerable role in facilitating our understanding of the GnRH phenotype. Although currently hampered by the experimental uncertainties and constraints common to the introduction of any new technology, the GnRH-GFP and GNZ mice should soon become routine tools in GnRH neurobiology laboratories. While we have shown that the GnRH neurons can be accessed without the need for transgenics, it is undoubtedly easier to use fluorescence-based identification. Used with care and appreciation of the possible pitfalls, thin brain slices maintained in vitro should allow any neuroscientist access to living GnRH neurons in their native environment. In addition, the use of nuclear-located transgene markers of the GnRH phenotype will greatly facilitate conventional dual labeling procedures on these neurons. Together with techniques enabling the rapid profiling of genes expressed by GnRH neurons, these new tools should facilitate the rapid characterization of the membrane and molecular properties of these cells. Current advances being made in the production of genetically-encodable molecules capable of signaling intracellular events will also open up new avenues for examining living native GnRH neurons in real time. Relating the cellular and molecular properties of these neurons to specific functions in vivo will be more demanding but, doubtless, also rely heavily on the genetic manipulability of the mouse.

Acknowledgements

We thank Sandra Dye for assistance with the mice. Work was supported by the UK Biotechnology and Biological Sciences Research Council and a European Travelling Fellowship from the Wellcome Trust (Jean-Remi Pape).

References

Amoss, M., Burges, R., Blackwell, R., Vale, W., Fellows, R., Guillemin, R., 1971. Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing hormone factor (LRF) of ovine origin. Biochem. Biophys. Res. Commun. 44, 205–210.

Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N., Langer, S.Z., 1998. International Union of Pharmacology XV Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev. 50, 291–313.

Barry, J., DuBois, M.P., Poulain, P., 1973. LRF producing cells of the mammalian hypothalamus. Z. Zellforsch. 146, 351–366.

Brussaard, A.B., Herbison, A.E., 2000. Long term $GABA_A$ receptor plasticity in the adult brain: insights from the oxytocin neuron. Trends Neurosci. 23, 190–195.

Cardenas, H., Ördög, T., O'Byrne, T., Knobil, E., 1993. Single unit components of the hypothalamic multiunit electrical activity associated with the central signal generator that directs the pulsatile secretion of gonadotropic hormones. Proc. Natl. Acad. Sci. USA 90, 9630–9634.

Chandran, U.R., DeFranco, D.B., 1999. Regulation of gonadotropin-releasing hormone gene transcription. Behav. Brain Res. 105, 29–36.

Feleder, C., Jarry, H., Leonhardt, S., Wuttke, W., Moguilevsky, J.A., 1996. The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinology 64, 305–312.

Herbison, A.E., Chapman, C., Dyer, R.G., 1991. Role of medial preoptic GABA system in regulating pulsatile luteinising hormone secretion in the ovariectomised rat. Exp. Brain Res. 87, 345–352.

Kawakami, M., Uemura, T., Hayashi, R., 1982. Electrophysiological correlates of pulsatile gonadotropin release in rats. Neuroendocrinology 35, 63–67.

Kepa, J.K., Spaulding, A.J., Jacobsen, B.M., Fang, Z., Xiong, X., Radovick, S., Wierman, M.E., 1996. Structure of the distal human gonadotropin-releasing hormone (hGnRH) gene promoter and functional analysis in GT1–7 neuronal cells. Nucl. Acids Res. 18, 3614–3620.

Krsmanovic, L.Z., Stojilkovic, S.S., Catt, K.J., 1996. Pulsatile gonadotropin-releasing hormone release and its regulation. Trends Endocr. Metab. 7, 56–59.

Kusano, K., Fueshko, S., Gainer, H., Wray, S., 1995. Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. Proc. Nat. Acad. Sci. USA 92, 918–3922.

Lira, S.A., Crenshaw, E.B. III, Glass, C.K., Swanson, L.W., Rosenfeld, M.G., 1988. Identification of rat growth hormone genomic sequences targeting pituitary expression in transgenic mice. Proc. Natl. Acad. Sci. USA 85, 4755–4759.

Mason, A.J., Pitts, S.L., Nikolics, K., Szonyi, E., Wilcox, J.N., Seeburg, P.H., Stewart, T.A., 1986. The hypogonadal mouse: reproductive functions restored by gene therapy. Science 234, 1372–1378.

Mellon, P.L., Windle, J.J., Goldsmith, P.C., Padula, C.A., Roberts, J.L., Weiner, R.I., 1990. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5, 1–10.

Mori, Y., Nishihara, M., Tanaka, T., Shimizu, T., Yamaguchi, M., Takeuchi, Y., Hoshino, K., 1991. Chronic recording of electrophysiological manifestation of the hypothalamic gonadotropin-releasing hormone pulse generator activity in the goat. Neuroendocrinology 53, 392–395.

Nelson, S.B., Eraly, S.A., Mellon, P.L., 1998. The GnRH promoter: target of transcription factors, hormones, and signaling pathways. Mol. Cell. Endocrinol. 140, 51–155.

Otis, T.S., Staley, I.J., Mody, I., 1991. Perpetual inhibitory activity in mammalian brain slices generated by spontaneous GABA release. Brain Res. 545, 142–150.

Pape, J.-R., Skynner, M.J., Allen, N.D., Herbison, A.E., 1999. Transgenics identify distal 5′- and 3′ sequences specifying gonadotropin-releasing hormone expression in adult mice. Mol. Endocrinol. 13, 2203–2211.

Pape, J.-R., Skynner, M.J., Sim, J.A., Herbison, A.E., 2001. Profiling $GABA_A$ receptor subunit mRNA expression in postnatal GnRH neurons of the male mouse with single cell RT-PCR. Neuroendocrinology, in press.

Quinn, J.P., 1996. Neuronal-specific gene expression—the interaction of both positive and negative transcriptional regulators. Prog. Neurobiol. 50, 363–379.

Radovick, S., Wray, S., Lee, E., Nichols, D.K., Nakayama, Y., Weintraub, B.D., Westphal, H., Cutler, G.B., Wondisford, F.E., 1991. Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. Proc. Natl. Acad. Sci. USA 88, 3402–3406.

Rubinstein, M., Liu, B., Goodman, R.H., Low, M.J., 1992. Targeted expression of somatostatin in vasopressinergic magnocellular hypothalamic neurons of transgenic mice. Mol. Cell. Neurosci. 3, 152–161.

Schally, A.V., Arimura, A., Baker, Y., 1971. Isolation and properties of the FSH and LH-releasing hormone. Biochem. Biophys. Res. Commun. 43, 393–399.

Schwanzel-Fukuda, M., Pfaff, D.W., 1989. Origin of luteinizing hormone-releasing neurons. Nature 338, 161–164.

Selmanoff, M., 1997. Commentary on the use of immortalized neuroendocrine cell lines for physiological research. Endocrine 6, 1–3.

Silverman, A., Livne, I., Witkin, J.W., 1994. The gonadotrophin-releasing hormone (GnRH) neuronal systems: immunocytochemistry and in situ hybridization. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1683–1706.

Sim, J.A., Skynner, M.J., Pape, J.-R., Herbison, A.E., 2000. Late postnatal reorganization of $GABA_A$ receptor signalling in native GnRH neurons. Eur. J. Neurosci. 12, 3497–3504.

Sim, J.A., Skynner, M.J., Herbison, A.E., 2001a. Heterogeneity in the basic membrane properties of postnatal GnRH neurons in the mouse. J. Neurosci. 21, 1067–1075.

Sim, J.A., Skynner, M.J., Herbison, A.E., 2001b. Direct regulation of postnatal GnRH neurons by the progesterone derivative allopregnanolone in the mouse. Endocrinology, 142, 4448–4453.

Simonian, S.X., Herbison, A.E., 2001. Differing, spatially-restricted roles of ionotropic glutamate receptors in regulating the migration of GnRH neurons during embryogenesis. J. Neurosci. 21, 934–943.

Simonian, S.X., Skynner, M.J., Sieghart, W., Essrich, C., Luscher, B., Herbison, A.E., 2000. Role of $GABA_A$ receptor $\gamma 2$ subunit in the development of the gonadotropin-releasing hormone neurons in vivo. Eur. J. Neurosci. 12, 3488–3496.

Skynner, M.J., Slater, R., Sim, J.S., Allen, N.D., Herbison, A.E., 1999. Promoter transgenics reveal multiple gonadotropin-releasing hormone-1-expressing cell populations of different embryological origin in mouse brain. J. Neurosci. 19, 5955–5966.

Spergel, D.J., Kruth, U., Hanley, D.F., Sprengel, R., Seeburg, P.H., 1999. GABA-and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurone in transgenic mice. J. Neurosci. 19, 2037–2050.

Suter, K.J., Song, W.J., Sampson, T.L., Wuarin, J.-P., Saunders, J.T., Dudek, F.E., Moenter, S.M., 2000. Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. Endocrinology 141, 412–419.

Terasawa, E., Fernandez, D.L., 2001. Neurobiological mechanisms of the onset of puberty in primates. Endocr. Rev. 22, 111–151.

Waschek, J.A., 1995. Transgenic targeting of neuroendocrine peptide genes in the hypothalamic-pituitary axis. Mol. Neurobiol. 10, 217.

Weiner, R.I., Wetsel, W., Goldsmith, P., Escalera de la Martinez, G., Windle, J., Padula, C., Choi, A., Negro-Vilar, A., Mellon, P., 1992. Gonadotropin-releasing hormone neuronal cell lines. Front. Neuroendocrinol. 13, 119.

Wierman, M.E., Fang, Z., Kepa, J.K., 1996. GnRH gene expression in neuronal cell lines. Trends Endocr. Metab. 7, 60–65.

Wilson, R.C., Kesner, J.S., Kaufman, J.-M., Uemura, T., Akema, T., Knobil, E., 1984. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology 39, 256–260.

Wolfe, A.M., Wray, S., Westphal, H., Radovick, S., 1996. Cell-specific expression of the human gonadotropin-releasing hormone gene in transgenic animals. J. Biol. Chem. 271, 20018–20023.

Wray, S., Grant, P., Gainer, H., 1989. Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc. Nat. Acad. Sci. USA 86, 8132–8136.

Wu, T.J., Gibson, M.J., Silverman, A.J., 1995. Gonadotropin-releasing hormone (GnRH) neurons of the developing tectum of the mouse. J. Neuroendocrinol. 7, 899–902.

Zakaria, M., Dunn, I.C., Zhen, S., Su, E., Smith, E., Patriquin, E., Radovick, S., 1996. Phorbol ester regulation of the gonadotropin-releasing hormone (GnRH) gene in GnRH-secreting cell lines: a molecular basis for species differences. Mol. Endocrinol. 10, 1282–1291.

Zhang, Y.-Z., Naleway, J.J., Larison, K.D., Huang, Z., Haugland, R.P., 1991. Detecting lacZ gene expression in living cells with new lipophilic, fluorogenic β-galactosidase substrates. FASEB J. 5, 3108–3113.

Zhen, S., Dunn, I.C., Wray, S., Liu, Y., Chappell, P.E., Levine, J.E., Radovick, S., 1997. An alternative gonadotropin-releasing hormone (GnRH) RNA splicing product found in cultured GnRH neurons and mouse hypothalamus. J. Biol. Chem. 272, 12620–12625.