Interneuron Diversity series: Molecular and genetic tools to study GABAergic interneuron diversity and function

Hannah Monyer¹ and Henry Markram²

¹IZN, Department of Clinical Neurobiology, University of Heidelberg, Heidelberg, Germany
²Brain and Mind Institute, EPFL, Lausanne, Switzerland

Structural and functional diversity of GABAergic interneurons has become increasingly central in our understanding of the elemental steps of information processing in the brain. The use of different molecular, electrophysiological and anatomical techniques has provided a wealth of new information regarding GABAergic interneurons over the past decade but it has also led to confusion regarding the number of subtypes of GABAergic interneurons. Combinatorial approaches that also consider multiple parameters seem now to offer renewed hope for finally clarifying the structural diversity of GABAergic interneurons. New molecular techniques have become a powerful tool for exposing the functional diversity of GABAergic neurons at the cellular, microcircuit and systems levels. This article reviews literature regarding molecular tools that have been used, or that appear promising for future attempts, to classify GABAergic interneurons. Some important limitations will also be indicated.

The Holy Grail in neuroscience is to understand how a finite set of genes gives rise to complex behavior. There are, however, numerous complex steps from genome to phenotype. Understanding the emergence of the phenotype requires knowledge at several levels, including transcriptional control, specific profiles of genes expressed in a specific neuron, and how each of the resulting mRNA transcripts contributes to the phenotype. The GABAergic system is fast becoming a key target for addressing these questions because of its immense anatomical, electrical and synaptic diversity. This article reviews approaches that are used to reveal which mRNAs are expressed in GABAergic neurons, as well as those that attempt to study the steps leading to this expression.

Some of the techniques that will be discussed here can be, and have been, applied equally well to other cell types, be they glutamatergic, serotonergic or dopaminergic neurons. Reverse transcriptase polymerase chain reaction (RT-PCR) studies, for example, have provided a wealth of information regarding differential gene expression in identified neurons. Other techniques, such as labelling of neuronal cell populations with an in vivo marker, can also be used for any neuronal cell type but are particularly useful when studying GABAergic interneurons or other rare cell types that cannot be easily identified in an acute slice preparation. Transgenic techniques aimed at alteration or ablation of specific genes have become standard but have not been used frequently for specific modifications in GABAergic cell populations.

Other reviews in this Interneuron Diversity series address the questions of GABAergic interneuron diversity and why the study of GABAergic interneurons is important. Numerous studies have indicated that GABAergic interneurons are key players in the generation of oscillatory activity in different frequency bands [1]. Distinct oscillatory patterns have been associated with different behavioural states. However, it is not known so far which genes determine the specific functional and morphological features of GABAergic interneurons that enable them to control the input and output properties of large ensembles of pyramidal neurons.

RT-PCR studies are crucial for identifying gene expression patterns in GABAergic interneurons. Labelling GABAergic interneurons with an in vivo marker permits systematic electrophysiological studies of different GABAergic interneuron subtypes. Finally, genetic manipulations restricted to subsets of GABAergic interneurons help to elucidate the function of specific genes at the cellular, network and behavioural levels.

Single-cell RT-PCR
Detecting specific mRNA transcripts in a single neuron is a major technical challenge, owing to the very small amounts of mRNA involved. A powerful technique that has been used to study ligand- and voltage-gated channels in GABAergic interneurons is single-cell RT-PCR. The most straightforward RT-PCR strategy is to use the cytoplasm of a cell to test for expression for a single gene [2–4]. A second approach is to split the cytoplasm into many reactions as genes to be tested before amplifying each gene independently [5,6]. A third strategy, useful when the genes of interest show a high degree of sequence similarity, is to design a single pair of degenerate primers for regions identical or nearly identical in all the genes [2,7–10]. Subsequently, the identity of each gene can be determined by differences in the size of the PCR products, by using specific restriction enzymes that cut only one
amplified PCR product or by second PCR reactions using gene-specific nested primers. A fourth method is non-specific pre-amplification of all mRNA of the neuron before the gene-specific PCR, using either PCR [11–13] or T7 mRNA polymerase [14–16]. mRNA amplification with T7 polymerase is routinely used for conventional DNA microarrays, where the starting amount of mRNA is large (~5 µg), but scaling this down to the picograms of mRNA available from a single neuron is a major challenge. A fifth strategy is based on gene-specific pre-amplification multiplex-PCR, which has allowed the detection of up to 15 expressed genes from a single neuron [17]. Each of these approaches suffers from serious limitations and all single-cell genetic approaches face the problem of a significant number of false negatives (genes expressed but not detected). Nevertheless, fundamental insight into the gene expression that underlies the different types of interneurons can be obtained if these limitations are directly addressed.

Technical aspects and protocols can be found in the papers and reviews cited in the preceding paragraph. It is beyond the scope of this review to attempt to discuss advantages and disadvantages of the different RT-PCR approaches. A direct comparison is impossible because it would require a systematic study performed in the same laboratory, addressing the same scientific question and analyzing the strengths and shortcomings of the different approaches. By reviewing the literature on RT-PCR, however, one notices that no reason is given why a particular approach was chosen, although in most cases the selection of the strategy was dictated by the scientific question. Thus, both the method developed by Eberwine and colleagues [14] and multiplex-PCR are certainly useful when detection of many different genes is of interest. However, when quantitative differences need to be analysed, this is most likely not the method of choice. In other words, to establish by single-cell RT-PCR whether a neuron is GABAergic [by the presence or absence of mRNA that is expressed only in that cell type, such as glutamic acid decarboxylase (GAD)65 or GAD67], it does not matter which strategy is chosen. By contrast, to amplify different genes of the same family and show that two cell types differ with respect to the relative amounts of mRNA expression requires an approach that guarantees faithful amplification of the starting material. Thus, when the analysis aims to evaluate the relative amount of the expressed subunits, primer selection and PCR conditions can require numerous control experiments to ascertain that the different subunits are amplified equally well independently of the starting ratio [18]. It is sometimes impossible to establish conditions that guarantee comparable amplification of two subunits, even if there is a high degree of nucleotide similarity in the region being amplified (H. Monyer, unpublished). In essence, studies where these controls have not been performed are questionable. Multiplex RT-PCR also suffers from the problem that different primers used for amplifying different genes can interact, requiring a lengthy calibration procedure in which the optimal combination and relative concentrations of primers must be determined.

Correlating molecular and functional studies in GABAergic interneurons
It should be emphasized that when the RT-PCR approach is used alone, the information obtained is descriptive at best, and in fact a carefully carried out in situ hybridization study is equally or even more informative. However, combining RT-PCR and electrophysiological studies in identified neurons yields information that is a prerequisite for subsequent studies aimed at manipulation of key genes and gaining a better understanding of network phenomena.

Some examples will help illustrate how RT-PCR was useful to correlate gene expression in GABAergic interneurons with specific functional properties. Single-cell PCR in combination with patch-clamp recording has for example, been successfully used to elucidate the function of differential glutamate AMPA receptor subunit (GluR) composition in subclasses of neurons. AMPA receptors are present in almost all neurons in the adult brain and mediate fast synaptic glutamatergic transmission. Molecular cloning revealed the presence of four receptor subtypes – GluR-A to GluR-D (also known as GluR1 to GluR4) – that are differentially expressed in the brain. The existence of a correlation between low GluR-B expression in GABAergic interneurons and Ca²⁺ permeability of AMPA receptors was clearly demonstrated in several studies employing single-cell PCR and patch-clamp recording [8,9].

Useful correlations were also obtained when studying AMPA receptor subunit expression and kinetic properties of the receptors: the fast deactivation and desensitization kinetics of AMPA receptors in GABAergic interneurons can be explained by expression of GluR-D and low expression of GluR-B [19,20].

It should be pointed out that in both cases, single-cell RT-PCR combined with electrophysiology confirmed a hypothesis based on in situ hybridization data and functional studies of recombinant receptors. This is not to diminish the importance of this method but some caution is warranted at this point: the method is powerful to substantiate a hypothesis and obtain correlative data but not to obtain absolute data.

Another example that illustrates the usefulness of the method regards the expression of K⁺ channels that account for a specific spiking pattern of a subset of GABAergic interneurons, the so-called fast-spiking cells. The spiking pattern of neurons has often been used as an electrophysiological signature that aids in identification of specific subclasses. In situ hybridization studies and functional analysis of recombinant K⁺ channels proposed the delayed rectifiers Kv3.1 or Kv3.2 as the molecular substrates that endow fast-spiking cells with the capacity to discharge at high rates upon prolonged depolarization. However, studies of recombinant channels also revealed that the voltage dependence and activation kinetics of Kv3.1 or Kv3.2 in heterologous systems differ from those of delayed rectifiers in fast-spiking cells. In the attempt to identify the channels that underlie fast-spiking, Surmeier and colleagues extended the single-cell PCR based expression analysis to other delayed-rectifier channels. They found that Kv3.4 was expressed in fast-spiking but
not in other neurons, and showed that Kv3.1 and Kv3.4 co-assemble to contribute towards fast-spiking [21].

The above examples illustrate the power of single-cell PCR when used in conjunction with electrophysiological studies. Both cases, albeit correlative in nature, strongly support the notion that there is a causal relationship between mRNA expression and specific channel properties. Only a few studies have attempted to strengthen the evidence for a causal relationship between the expression of a single ion channel gene and the electrical behavior, using for example dynamic clamp [22], antisense oligonucleotides [23], transfection with dominant-negative mutants [24], single-cell pharmacology and transfection of human embryonic kidney (HEK) cells [21], and modelling of neocortical neurons combined with immunohistochemical and pharmacological approaches [25].

A major problem in current single-cell RT-PCR approaches is the use of the ‘frequency of expression’ to evaluate the correlation between the expression of a gene and a particular electrophysiological behaviour. This is a problem because any value <100% suggests largely methodological flaws either in detecting the gene or in returning to the same cell type. Another difficulty in correlating expression of individual genes with electrophysiological behaviour is the fact that each gene works in concert with >100 ion channel genes to shape specific or global electrical properties. A new approach addresses both of these issues [26] (M. Toledo-Rodríguez and H. Markram, unpublished). This approach employs multiplex RT-PCR (the expression of 50 genes was analyzed) (Figure 1) and a large number of cells in an attempt to quantify the correlation between the expression (or non-expression) of genes and parameters of electrophysiological behaviour. These parameters include, for example, the amplitude and duration of the action potential and the afterhyperpolarization, the rate of spine train accommodation and discharge rates. The expression (or non-expression) of a gene can then be correlated with specific electrophysiological values. The derived correlation coefficient can be used to predict the values of a spectrum of electrical properties even from non-quantitative PCR data. Conversely, the probability that any of the 50 genes is expressed can be calculated by measuring only the electrical parameters. A major strength of this approach is that it directly addresses the problem of methodological flaws, especially false negatives, by using very large datasets to carry statistical modelling. The manner in which sets of genes can be correlated to electrophysiological behaviour can also be determined. Future studies could combine such an approach with multiple RNA silencing to determine the extent to which the correlations translate in to causal effects, potentially making it possible to establish the causal relationship between the genes expressed and the emergent GABAergic interneuron phenotype.

Transgenic techniques – altering gene expression in GABAergic interneurons

All studies that have demonstrated either the presence or the absence of certain proteins in GABAergic interneurons invariably lead to the question of why GABAergic interneurons require the presence of a particular protein or, conversely, to that of whether the absence is functionally important. A major advance in the pursuit of such questions has been the use of transgenic techniques.

The finding that AMPA receptor properties in GABAergic interneurons are attributable in large measure to low GluR-B expression was, for example, taken from a phenomenological or cellular level to the system level by overexpressing this subunit selectively in GABAergic interneurons [27]. This study was prompted by modelling data suggesting that fast kinetics of AMPA receptors in GABAergic interneurons are crucial for long-range synchronous neuronal activity. Indeed, as predicted by the modelling data, overexpression of GluR-B in GABAergic interneurons led to a prolongation of excitatory postsynaptic potentials (EPSPs) in interneurons that is likely to account for loss of long-range synchronous oscillatory activity between two tetanically stimulated sites in the hippocampus.

Selective gene ablation in GABAergic interneurons has not been attempted so far but has been achieved to some extent by global knockout approaches because certain genes show a preferential expression in GABAergic interneurons. Thus, Kv3.1, connexin 36 and GluR-D, to name just a few, are expressed not exclusively but preponderantly in GABAergic interneurons, at least in some brain areas. The knockout mice have helped in part to understand why their expression in GABAergic interneurons is relevant at the network level [28,29].

Paul and colleagues combined the connexin36 knockout mouse with the concomitant knockin of the marker gene lacZ (encoding β-galactosidase), to permit easy localization of cell types normally expressing the gene [28]. Such an approach is particularly useful when expression studies are hampered by the lack of specific antibodies. The combination of knocking out or overexpressing a gene and the simultaneous marking of the cell type in which such modifications occur had been previously reported. This was achieved by introducing an internal ribosome entry site (IRES)-lacZ cassette in the to-be-modified gene [30,31]. Strong promoters ensure the labelling not only of cell bodies but also of axons, thus permitting axon-targeting studies. This was successfully used, for example, in studies where IRES-tau-lacZ was introduced into olfactory neuron receptor genes [32]. However, the IRES cassette is often associated with a diminished expression of the subsequent marker gene, be this lacZ or a gene encoding green-fluorescent protein (GFP) (H. Monyer, unpublished).

Making use of the Cre-lox system [33] will be helpful to limit gene ablation to GABAergic interneurons or even to particular subsets of these neurons. Selective knockout of key molecules expressed in GABAergic interneurons will be required to demonstrate their functional importance not only at the cellular level but also at the network level, and for behaviour. Pharmacological tools are not helpful in substantiating models that require selective inactivation of proteins in GABAergic interneurons only, if these proteins are present in other neurons as well. For example, based on experimental and modelling data, AMPA receptor activation in GABAergic interneurons is required for the induction of oscillatory gamma frequency activity in the CA3 region of the hippocampus. An AMPA receptor
antagonist will not prove useful because AMPA receptors in interneurons and pyramidal cells alike will be blocked. However, in mice expressing Cre recombinase in GABAergic interneurons and harboring the ‘floxed’ GluR-A receptor gene, AMPA-receptor-mediated currents should be reduced selectively in GABAergic interneurons (Figure 2).

It is beyond the scope of this review to discuss the importance of computer modelling data (see Ref. [1]) that help in the ‘design’ of useful transgenic mice. Many mouse lines generated in the laboratory of H. Monyer take into account predictions based on computer models.

In vivo labelling of GABAergic interneurons and circuits
A powerful molecular tool for studying GABAergic interneurons functionally is the use of modified bacterial artificial chromosomes (BACs) to generate transgenic mice expressing the in vivo marker GFP. The relatively easy modification of BACs by homologous recombination in Escherichia coli and the subsequent use to generate transgenic animals were first described by Heintz and colleagues [34]. BACs have the advantage of containing long pieces of genomic DNA, increasing the likelihood of correct temporal and spatial control of gene expression.

This method has proved to be extremely useful for labelling subsets of GABAergic interneurons (H. Mon- yer, unpublished) but it must be emphasized that the use of BACs is not a guarantee for the correct expression of the transgene. The generation of transgenic mice expressing GFP under the control of the parvalbumin promoter was a successful enterprise [34]: the parvalbumin promoter on the modified injected BAC ensures the correct expression of GFP in most parvalbumin-positive cells. However, the use of BACs still requires detailed expression analysis of the transgene. This is not a problem when specific antibodies or reliable riboprobes are available but can turn out to be difficult when this is not the case.

Once the correct expression of GFP in subsets of GABAergic interneurons is ascertained, the transgenic mice are highly useful for several functional studies. In transgenic mice expressing GFP under the control of the parvalbumin promoter, recordings could be made easily from pairs of labelled neurons in slices from adult animals, to investigate the frequency and strength of electrical coupling between parvalbumin-positive cells in different brain regions [35]. Normally such studies are extremely difficult, if not impossible, because the identification of specific GABAergic interneurons is time consuming when based on the spiking pattern, and the condition of the slices from adult animals deteriorates rapidly. In slices where specific interneurons are fluorescently labelled, no time need be spent on cell-type identification, permitting functional studies of rare cell types, even in slices obtained from adult mice.

In addition, transgenic mice with labelled subsets of GABAergic interneurons allow systematic study of the labelled neurons. Because the study of large numbers of neurons is feasible, one can ask questions regarding the homogeneity of such neurons in different brain regions. Thus, in slices obtained from transgenic mice with GFP-labelled parvalbumin-positive neurons, one can easily investigate how similar parvalbumin-positive neurons are in distinct areas such as hippocampus, visual cortex or somatosensory cortex. Recording from 'green cells' in slices from transgenic parvalbumin-GFP mice has allowed a new subtype of parvalbumin-positive neurons, the multipolar bursting cells, to be identified [36]. These GABAergic interneurons differ from the 'fast-spiking' parvalbumin-positive neurons in all aspects investigated: they exhibit distinct anatomical, biochemical and functional features and, most importantly, have a distinct function at the network level (Figure 3).

Studies of in vivo labelled GABAergic interneurons are only starting but transgenic mice in which subsets of GABAergic interneurons are fluorescently marked are a precious tool for researchers focusing on these neurons. In the study by Blatow and colleagues [36], the systematic study of labelled parvalbumin-positive neurons revealed that a heterogeneous cell population can be subdivided into well-defined classes of GABAergic interneurons with a unique combination of morphological, pharmacological and physiological properties.

Using transgenic techniques, it is possible to label not only subsets of cells but also proteins [37]. As with the former, the study of crucial proteins in GABAergic interneurons is slightly more complicated than in other cells, because specific expression in GABAergic interneurons requires the availability of promoters that ensure differential protein expression in GABAergic neurons or even in subsets thereof. Here too, BACs containing specific promoters could ensure the correct expression of the chimeric proteins.
Figure 3. Discovery and characterization of a new parvalbumin-positive interneuron subtype – the multipolar bursting cell (MB) – using transgenic mice expressing enhanced green-fluorescent protein (EGFP) under control of the parvalbumin promoter. Multipolar bursting cells can be distinguished from the majority of parvalbumin-positive cells (fast-spiking cells [FS]) by several criteria. (a) A green-fluorescent cell in cortical layer (L) 2/3, and parvalbumin (PV) immunocytochemistry indicating the correct transgene expression. Red and blue indicate dendrites and axons, respectively. Scale bar, 20 μm. (b) Morphological reconstruction and spiking pattern of fast-spiking and multipolar bursting cells. Scale bar, 100 μm. (c) Fast-spiking cells exhibit paired-pulse depression, in contrast to multipolar bursting cells, which show paired-pulse facilitation. Red traces indicate inhibitory postsynaptic potentials in pyramidal neurons (Pyr) elicited by action potentials in fast-spiking cells (black traces). (d) Different ‘working frequencies’ of the two cell types upon sinusoidal current injection. Traces on the left are from fast-spiking cells; traces on the right are from multipolar bursting cells. Modified, with permission, from Ref. [36].

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Although identification of GABAergic interneurons in live tissue is time-consuming, functional studies of more abundant cell types (e.g. parvalbumin-positive neurons) have been feasible. The study of rare cell types (e.g. interneurons expressing calretinin or nitric oxide synthase), and particularly their functional connectivity, is indeed a challenge. One possible solution to this problem would be to generate mice in which a fusion protein (tau-GFP) marks the cell body and axons of such neurons, to aid in the identification not only of these rare neurons themselves but also of their target cells.

Systematic in vivo studies of identified interneurons have not been feasible so far. In an elegant study by Klausberger and colleagues [38], the activity of three types of interneurons was correlated with network oscillatory activity. The identity of interneurons could be established only after filling the neuron and sacrificing the animal. Needless to say, such studies are extremely demanding and will profit in the future from mouse lines with specifically labelled subsets of interneurons. A further step will entail in vivo recordings from labelled interneurons in the live animal, to correlate cellular activity of identified neurons with behaviour.

Alteration and silencing of GABAergic interneuron activity

An interesting approach for modifying GABAergic interneuron activity is being pursued by Wisden and colleagues [39]. If successful, this will be a very powerful tool to study the function of different GABAergic interneurons at the system level. The method is a combinatorial multistep approach between genetic and pharmacological manipulation of GABAergic interneurons and is based on the finding that modulation of GABA_A receptors by benzodiazepines depends on the presence of a single amino acid residue in the γ2 subunit of the receptor. In genetically altered mice bearing a mutation that renders all GABA_A receptors benzodiazepine-insensitive, a wild-type γ subunit is reintroduced in selective cell populations, for instance in GABAergic neurons or subsets thereof. In such genetically altered mice, GABA_A receptor neurotransmission would not be affected but could be pharmacologically increased or decreased selectively in neurons bearing the wild-type γ subunit.

Of note are also diverse attempts aimed at the complete or functional ablation of selected cell populations. Complete ablation of specific cell types (e.g. starburst amacrine cells in the retina or Golgi cells in the cerebellum) has been reported by Nakaniishi and colleagues [40]. The method entails the generation of transgenic mice expressing the α subunit of the interleukin 2 receptor in a selected cell population. Ablation of the neurons expressing the transgene is achieved by local injection of exotoxin-coupled antibodies to the receptor. One advantage of the method is the possibility to ablate specific cell populations in the adult, thus avoiding the problem that many transgenic manipulations bear, of interfering at early developmental stages. A disadvantage is the necessity of local injection of the antibodies.

Another approach, taken by Callaway and colleagues, is the functional silencing of neurons via activation of an insect G-protein-coupled receptor known to activate certain mammalian inward-rectifier K⁺ (GIRK) channels [41]. Application of the peptide ligand leads to hyperpolarization of the cell membrane and hence to silencing of neuronal activity. The beauty of this system lies in its specificity – the ligand apparently activates only neurons expressing the insect G-protein-coupled receptor – and reversibility. However, the experiments were performed in slices and it remains to be seen whether the approach is applicable in vivo. This will certainly depend on sufficient expression levels of the transgene (the insect G-protein-coupled receptor) and GIRK channels, as well as correct expression of the transgene in the neuronal cell type to be silenced. The application of the peptide ligand in vivo would have to be via local injection. Lester and colleagues aim to silence neurons by expressing an invertebrate glutamate-activated Cl⁻ channel in them; here too, positive results have been reported so far only in cell culture [42].

Considering that any of the current approaches for studying GABAergic interneurons using light microscopy, electron microscopy, or electrophysiological or molecular techniques are difficult and highly time-consuming, it is not surprising that the systematic study of GABAergic interneurons is a daunting task. A certain dejection is inevitably reflected in articles entitled ‘How many subtypes of inhibitory cells in the hippocampus?’ [43]. However, significant progress has been made in recent years and the use of new molecular techniques and systematic combinatorial approaches should help to clarify the current picture rather than contribute to a greater confusion.

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