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Correlating function and gene expression of individual basal ganglia neurons

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Functional studies at the level of individual neurons have greatly contributed to our current understanding of basal ganglia function and dysfunction. However, identification of the expressed genes responsible for these distinct neuronal phenotypes is less advanced. Qualitative and quantitative single-cell gene-expression profiling, combined with electrophysiological analysis, allows phenotype–genotype correlations to be made for individual neurons. In this review, progress on gene-expression profiling of individual, functionally characterized basal ganglia neurons is discussed, focusing on ion channels and receptors. In addition, methodological issues are discussed and emerging novel techniques are introduced that will enable a genome-wide comparison of function and gene expression for individual neurons.

Electrophysiological and anatomical analysis of individual neurons in the basal ganglia circuitry has been instrumental in identifying their functional roles and computational contributions within the basal ganglia network [1–3]. In addition, studies that revealed the changes of single-cell activity in the dopamine-depleted basal ganglia have refined our understanding of the pathophysiological processes in basal ganglia disorders [4,5]. These neuronal phenotypes have been compiled not only in rodent and primate models but also in human patients [4,6]. By contrast, the identification of genes that are responsible for these physiological and pathophysiological phenotypes of basal ganglia neurons is less advanced. Ideally, the gene expression of functionally and anatomically identified neurons should be studied with highest possible resolution – at the level of the individual cell. This short review discusses recent progress in gene-expression profiling of individual, functionally characterized basal ganglia neurons and provides an outlook on emerging single-cell functional genomics approaches.

Selective harvesting and specific amplification of single-neuron mRNA

Methods for analyzing phenotype–genotype correlations of individual neurons were pioneered in the early 1990 s [7–9]. In essence, these techniques combine electrophysiological whole-cell patch-clamp recordings of individual neurons with subsequent gene-expression profiling. Figure 1 provides an overview of the main strategies for detecting mRNA from single cells. Briefly, after electrophysiological characterization, cytoplasm is harvested via the patch pipette, or the whole cell is aspirated. Subsequently, complementary DNA (cDNA) is synthesized without a separate RNA isolation step. Optionally, to avoid genomic DNA contaminations (Figure 2), a DNase digest can be performed before reverse transcription (RT). After amplification of single-cell cDNA via PCR-based or antisense RNA (aRNA) amplification techniques, gene expression can be analyzed (e.g. by PCR-based or microarray-based approaches). It is beyond the scope of this review to discuss general methodological aspects in detail, the reader is referred to recent reviews [10–12]. However, some crucial issues are highlighted here.

One difficulty of such approaches for detecting mRNA from single cells is how to interpret negative signals. Ideally, the absence of a signal should correspond to no expression of the respective gene in the analyzed cell. But technical problems (e.g. RNase contamination, or variations in efficiency of harvesting, RT or PCR) might easily confound this simple interpretation. Owing to the enormous amplification necessary to detect a few mRNA molecules from single neurons, it is essential to ensure that the detected gene expression is specific for the investigated neuron. In RT-PCR-based approaches, false-positive signals could be generated from the genomic DNA (i.e. the two gene copies within the neuronal nucleus). The best way to differentiate between genome-derived and mRNA-derived PCR signals is to design intron-spanning primers, which also identify unspliced heteronuclear RNA by size. If this is not possible (e.g. for intronless genes) one has to individually test each primer set for the possibility of genomic PCR amplification, for example by omitting reverse transcriptase in the RT-PCR reaction (an RT-minus control; Figure 2). In case of 'false-positive' PCR signals, derived from genomic DNA, a DNase digest needs to be carried out before RT-PCR. Figure 2 illustrates this strategy for the single-cell RT-PCR analysis of dopamine receptor (D1–D5) expression [13]. Primers for D1 and the intronless D5 were not intron-flanking, and without

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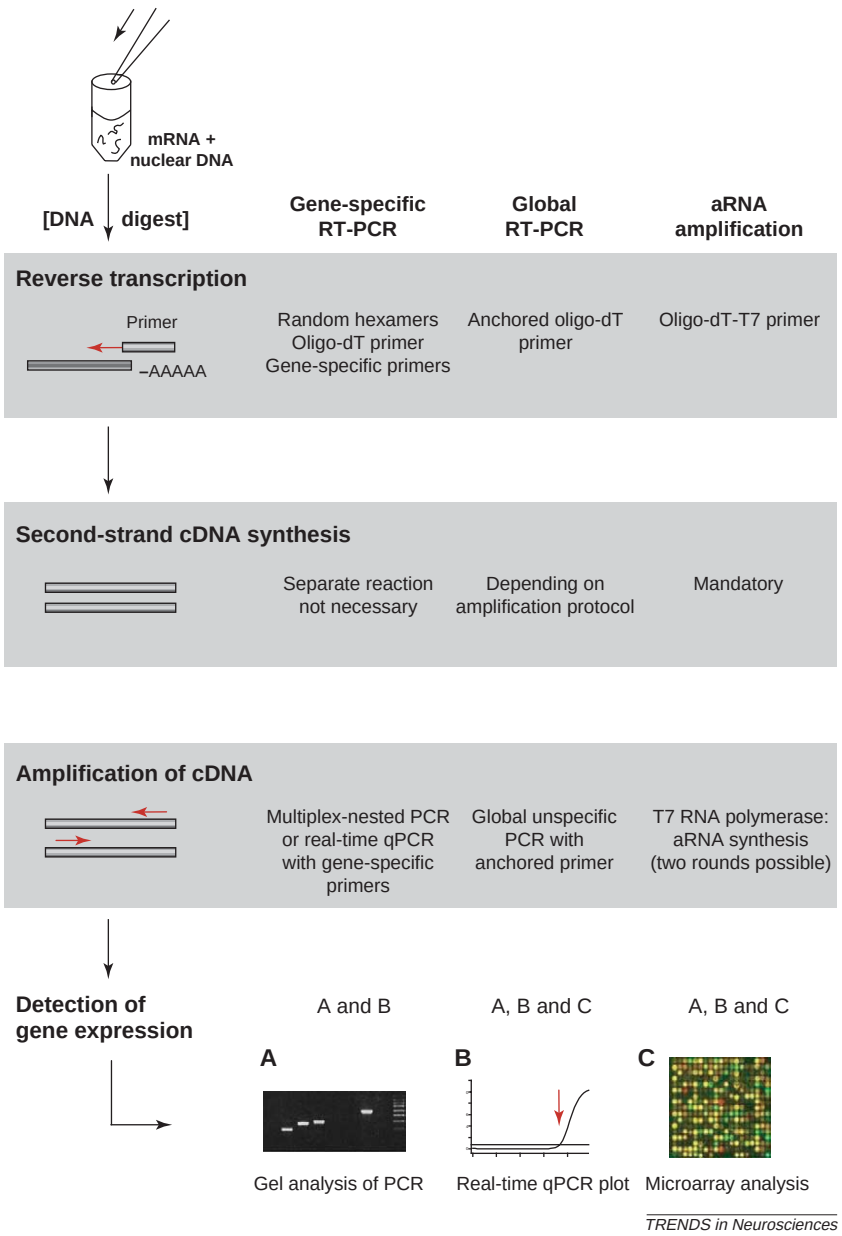

Figure 1. Principle of single-cell gene-expression techniques. After harvesting of single cells or single-neuron cytoplasm via a patch pipette, contents are expelled into a reaction tube; no separate RNA isolation is required. For gene-specific reverse transcription (RT)-PCR, mRNA is reverse transcribed into first-strand complementary DNA (cDNA) with random hexamers, oligodeoxy-thymidine (oligo-dT) or gene-specific primers. First-strand cDNA is directly used as template either for two rounds of qualitative PCR (multiplex-nested PCR), or for quantitative (real-time) PCR (qPCR), utilizing gene-specific primers. For global RT-PCR amplification, mRNA is reverse transcribed with oligo-dT primers containing a specific anchor sequence. Some global amplification protocols require second-strand cDNA synthesis and/or 5′-end tailing before global PCR amplification with anchored primers. For antisense RNA (aRNA) amplification, first-strand cDNA is synthesized using oligo-dT primers that contain a T7-RNA-polymerase-binding sequence. After second-strand cDNA synthesis, antisense RNA is generated via RNA polymerase, leading to ~1000-fold amplification of single-cell cDNA. In most cases, this aRNA is used for a second round of cDNA synthesis and aRNA amplification, resulting in ~10^6-fold amplification. Products of global or aRNA amplification can be directly quantified, or used either for fluorescence labelling and microarray hybridization or as templates for gene-specific qualitative or quantitative PCR. Detection of single-cell gene expression is performed via gel electrophoresis of (nested) PCR products (A), analysis of real-time PCR fluorescence plots (B) or analysis of microarray scans (C).

DNase digest, PCR signals were generated in all analyzed single cells, including the RT-minus control.

Expression profiling of single neurons in the basal ganglia

In the basal ganglia, aRNA-based and RT-PCR-based variants of single-cell expression profiling were pioneered by Jim Surmeier and colleagues to analyze the expression of the five different dopamine receptors in distinct sub-populations of striatal projection neurons [14,15]. This was of particular interest because the segregation of D1-class and D2-class dopamine receptors to striatonigral and striatopallidal subpopulations was considered to be a key element in the functional anatomy of direct and indirect networks in the basal ganglia. The aRNA-based study indicated, however, that D1-class and D2-class receptors were coexpressed and colocalized in many striatonigral neurons (identified via retrograde tracing). The later RT-PCR based study confirmed these findings. About 50% of striatonigral neurons (identified via substance P

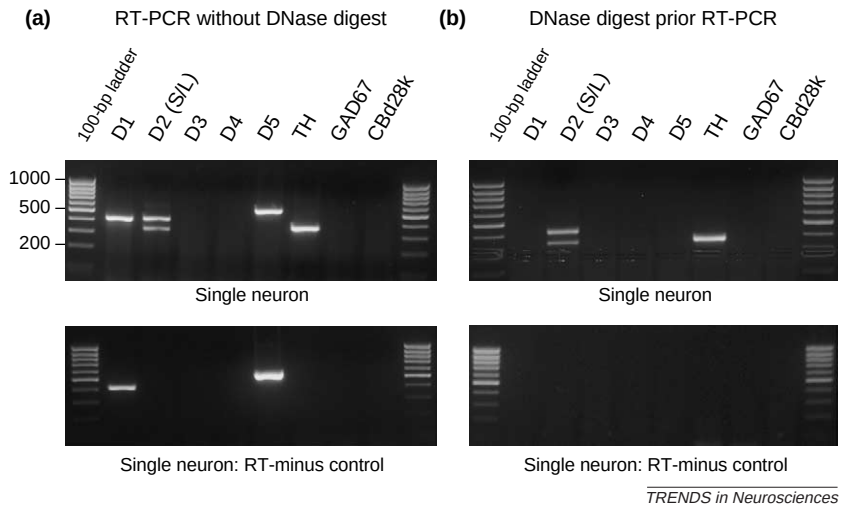

Figure 2. Single-cell reverse transcription (RT)-PCR for dopamine receptors with and without DNase digestion. Gel electrophoresis of multiplex-nested PCR products (five dopamine receptors and three markers) derived from four different dopaminergic midbrain neurons. Primers for D1 and D5 dopamine receptors were not intron-spanning. Lower panels: to probe for 'false positive' RT-PCR signals derived from the genomic DNA of the nucleus, the reverse transcriptase was omitted in these RT-PCR reactions. **(a)** RNA from cytoplasm (including nucleus) of single cells was directly reverse transcribed. Note that, in contrast to D2 receptor and tyrosine hydroxylase (TH) signals, D1 and D5 signals were amplified for both single neurons, including the RT-minus control cell. These results make it impossible to decide whether the D1 and D5 signals in the upper panel were derived from mRNA or genomic DNA. **(b)** RNA from cytoplasm (including nucleus) of single cells was subjected to DNase digest before reverse transcription. Note that signals for only D2 receptors and TH are detected for the single neuron shown in the upper panel; D1 and D5 signals were absent. After DNase digest, the RT-minus control was blank. These results suggest that the signals for D1 and D5 for the neuron in (a), upper panel were false-positive results derived from genomic DNA. Abbreviation: CBd28k, calbindin; D1–D5, dopamine receptors (S/L, short and long splice variants); GAD67, glutamic acid decarboxylase 67; TH, tyrosine hydroxylase.

and enkephalin mRNA coexpression profiles) expressed D1 receptors as well as D3 or D4 receptor mRNA.

These early studies illustrate some major advantages of the single-cell RT-PCR technique: (i) high resolution, which allows analysis and definition of specific neuronal subtypes that might be present in only a small fraction of the total neuronal population (assessed as a collective with tissue-based methods); (ii) identification of the molecular phenotype of the analyzed neuron via co-detection of marker mRNA (e.g. that encoding enzymes for neurotransmitter synthesis, neuropeptides or Ca^{2+}-binding proteins); and (iii) very high sensitivity, which enables the detection of even one or two target molecules. This sensitivity is further illustrated in Figure 2 by the detection of two genomic DNA copies from a single cell nucleus. Detection of complex, often unexpected mRNA coexpression profiles such as those for dopamine receptors in striatal neurons created novel insights into the molecular diversity of neuronal populations and raised important questions regarding the functional significance of these mRNA coexpression patterns.

However, these studies also indicate potential limitations of single-cell approaches. Neurons were acutely dissociated following enzyme treatment. This procedure could dramatically change their morphology and results in a loss of local environment, which might alter their functional properties and gene expression. There is also danger of creating a non-representative selection of cell types that are less affected by the isolation procedure. These problems are alleviated when using brain-slice preparations from young animals. However, many studies still rely on acutely dissociated neurons – probably because harvesting of cytoplasm from neurons in brain slices is also problematic as they are tightly surrounded by neuronal and glial neighbours, increasing the risk of contamination and unspecific signals. This risk is monitored by analyzing the expression of glial and cell-type-specific neuronal markers, and it can be minimized if the gigaseal is stable throughout the harvesting procedure [16]. Recently, single-cell RT-PCR was extended to neurons from slices of adult rodents, identified via retrograde tracing (Figure 3; B. Liss and J. Roeper, unpublished). This will enable expression profiling of single neurons in brain slices from well-established rodent basal ganglia disease models [17].

Another crucial advantage of combining electrophysiological and molecular single neuron analysis is the potential to study functional and pharmacological phenotypes (e.g. specific synaptic currents or selective drug responses) in combination with gene-expression profiles in the same neuron. This allows non-biased analysis of neurons within the different nuclei of the basal ganglia, without implying their homogeneity – a constraint of tissue-based approaches. As somatodendritic ion channels and receptors orchestrate the basic electrophysiological properties of basal ganglia neurons, most single-cell studies focused on the functional and molecular analysis of these signaling molecules.

Klink and colleagues compared nicotinic ACh (nACh) receptor currents induced by fast ligand application with expression profiles of subunits for the nACh-receptor family. They found for dopaminergic midbrain neurons a clear single-cell phenotype–genotype correlation between distinct current kinetics and the detection or non-detection of α7 nACh-receptor mRNA [18]. Sergeeva and Haas defined the expression profiles of glycine receptors for cholinergic forebrain interneurons [19]. Their elegant single-cell phenotype–genotype correlation revealed that

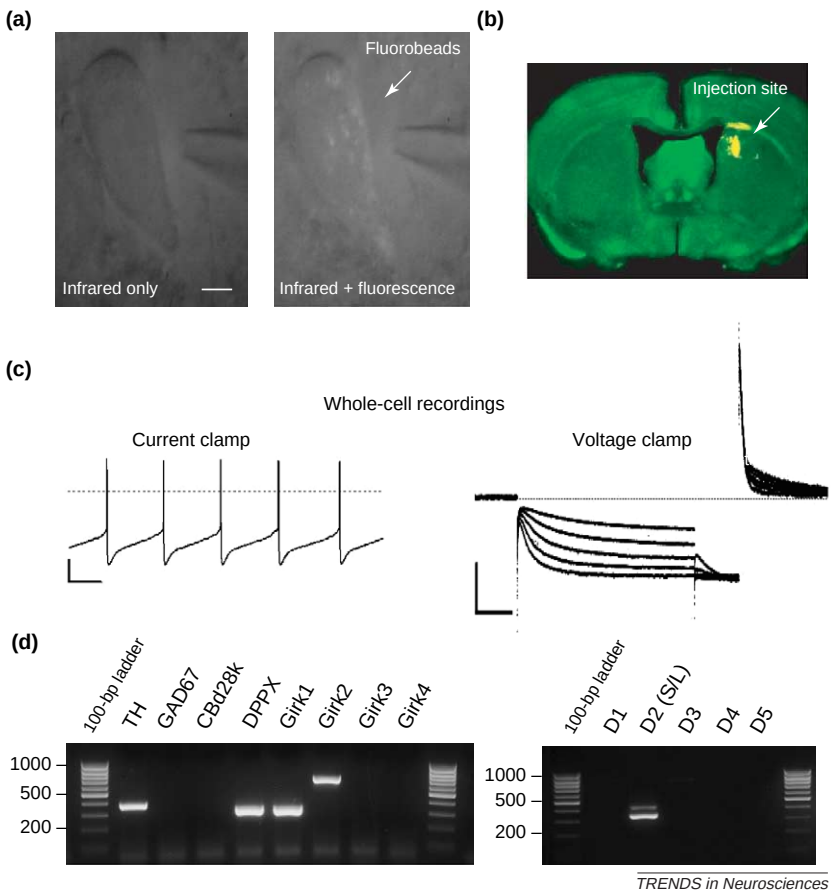

Figure 3. Single-cell reverse transcription (RT)-PCR of an identified mesostriatal dopaminergic neuron in adult mouse brain slices. **(a)** Infrared differential interference contrast (IR-DIC) video microscopy of a retrogradely labeled mesostriatal neuron. Fluorescence beads are visible (arrow) under fluorescent illumination (546 nm); scale bar, 4 μm. **(b)** Fluorescent green Nissl stain (488 nm) of a coronal brain section from the same animal as in (a) for verification of the dorsal medial striatal injection site of the retrograde tracer beads (546 nm). **(c)** Electrophysiological properties of the neuron shown in (a): current-clamp recording of spontaneous activity (scale bar, 25 mV and 500 ms) and voltage-clamp recording of ionic currents (holding potential −40 mV, with 2 s voltage steps of increasing amplitudes from −60 mV to −140 mV in steps of 20 mV, followed by 0.5 s at −140 mV; scale bar 500 pA and 400 ms). **(d)** Gel electrophoresis of gene-specific RT-PCR products (multiplex-nested). After recordings, cytoplasm was harvested via patch pipette. Resulting cDNA was split to analyze expression of 13 different genes (in sets of eight and five). Abbreviations: CBd28k, calbindin; D1–D5, dopamine receptors (S/L, short and long splice variants); DPPX, dipeptidyl peptidase 6; GAD67, glutamic acid decarboxylase 67; Girk1–Girk4, G-protein-coupled inwardly rectifying K^+ channel subunits; TH, tyrosine hydroxylase.

differential expression of the α3 subunit in mouse, but not in rat, cholinergic interneurons was correlated with differential desensitization kinetics of the native glycine receptors and with differential potency of the receptor agonist taurine.

However, robust single-cell phenotype–genotype correlations are difficult to identify – not only because of methodological considerations and the large number of possible channel and receptor candidate genes, but also because of their dynamic translation and assembly, differential presynaptic, postsynaptic and extrasynaptic targeting, and turnover of distinct hetero-oligomers within single cells [20]. In addition, in many studies the potential of genuine single-cell phenotype–genotype correlations were not fully exploited. For instance, individual phenotype–genotype correlations of distinct dopamine receptor coexpression were not fully resolved for striatal neurons [15], or function and gene expression were not analyzed in the same cells [21,22]. Baufreton and colleagues, by contrast, have recently demonstrated that neurons in the subthalamic nucleus that showed dopamine-induced burst-potentiation selectively expressed D5 but not D1 receptors.

Dopamine receptor activation alters the responsiveness of basal ganglia neurons by modulation of both synaptic efficiency and postsynaptic conductances [23,24]. Thus, there is considerable interest in identifying the molecular downstream targets for dopamine receptors. D2 receptors directly activate native G-protein-coupled inwardly rectifying K^+ channels that assemble from Kir3.1–Kir3.4 subunits (GIRK1–GIRK4). The expression of these GIRK channel subunits has been studied in particular for dopaminergic and GABAergic midbrain neurons [22,25,26]. In these studies, differential coexpression patterns of GIRK1–GIRK4 subunits were detected; however, their specific functional roles were not resolved. By contrast, Cruz and colleagues showed that distinct GIRK channel coexpression profiles between GABAergic and dopaminergic midbrain neurons were associated with differential coupling efficiencies of $GABA_B$ receptors to these K^+ channels [27]. Yan and Surmeier studied single-cell mRNA expression profiles of $GABA_A$ receptor subunits in striatal

cholinergic interneurons in the context of their finding that D5 receptor activation mediated an enhancement of $GABA_A$ currents [28]. In most choline-acetyltransferase-positive cells, a complex coexpression pattern, including the detection of α2, α4, β1 and γ2 nACh receptor subunits, emerged. In addition, α3, β3, γ1 and/or γ3 nACh receptor subunit mRNA was detected in many analyzed neurons. Possible functional implications of the differential subunit expression were not addressed.

Kir2.1–Kir2.3 (IRK1–IRK3) channel subunits form another class of inwardly rectifying K^+ channels that might be activated by D1 dopamine receptors in striatal neurons [29]. Kir2.1 detection was correlated by Mermelstein and colleagues with inactivation properties of inwardly rectifying K^+ currents in medium spiny neurons that coexpress distinct neuropeptide mRNA profiles [30]. Possible functional implications for cell-type-dependent diversity of up-state and down-state transitions in striatal neurons remain to be addressed.

Quantifying gene expression levels in basal ganglia neurons

Voltage-gated Ca^{2+} channels are also important downstream targets of D1-class and D2-class dopamine receptors [15]. Again, Mermelstein and colleagues [31] demonstrated for striatal medium spiny neurons that differences in expression levels of the Ca^{2+} channel β subunits 2a and 2b were associated with either slow or fast inactivation properties of Q-type Ca^{2+} currents.

To analyze relative differences in mRNA levels, the authors utilized a semi-quantitative single-cell RT-PCR approach previously developed by Tkatch and colleagues [32]. This method is based on generating serial dilutions of the reverse transcribed single-cell cDNA to determine the limiting dilution, where a respective PCR signal is still detected. In these serial dilutions, detection frequencies of individual transcripts were well described by unimodal Gaussian distributions, which facilitated statistical analysis and comparison across different neuronal populations [33–35]. The introduction of semi-quantitative single-cell RT-PCR methods was a significant improvement compared with the previous qualitative approaches. Qualitative PCR, in contrast to (semi-) quantitative PCR or aRNA amplification, is blind to biologically relevant differences of gene expression or respective mRNA levels and allows only a binary read-out: expression or absence of a distinct mRNA. An aRNA-based single-cell expression study analyzing $GABA_A$ receptors in hippocampal granule cells demonstrated the potential of quantifying gene expression levels via linear aRNA amplification approaches. The authors defined a complex pattern of $GABA_A$ receptor upregulation and downregulation in an epilepsy model [36]. Similarly, in basal ganglia nuclei, specific disease-related and plasticity-related changes in expression levels of signalling molecules that are not detectable with qualitative RT-PCR are likely to be relevant.

Analysis of single-cell expression profiles of ionotropic glutamate receptors were analyzed in great detail in other brain regions [7,37–41] but have not yet received similar attention in the basal ganglia. However, the important issue of differential expression and editing of the AMPA receptor GluR2 subunits, and the relevance of this for Ca^{2+} permeability of native AMPA receptors, has been addressed for single striatal neurons. Kim and colleagues found that NADPH-diaphorase-positive neurons are highly vulnerable to AMPA excitotoxicity and possessed a lower degree of RNA editing (at the Q/R site) [42]. This study also aimed to define the GluR1-to-GluR2 expression ratio in single striatal neurons by quantifying the relevant PCR product after 40 cycles of multiplex PCR using degenerate primers for the AMPA receptor family followed by 40 cycles of nested PCR for individual subunits. However, it would require an extremely well-established and carefully controlled protocol to obtain reliable and meaningful quantitative data via endpoint quantification of PCR products.

For quantitative RT-PCR based genotype–phenotype correlations of single neurons, fluorescence-based 'real-time' quantitative PCR approaches [43,44] might be the method of choice. For real-time PCR, it is crucial to remove factors (e.g. from the RT reaction) that distort the amplification kinetics, and consequently quantification. Thus, if utilizing the whole single-cell cDNA reaction as a real-time PCR template, a purification step might be necessary [45]. Real-time quantitative PCR was first applied to different types of single basal ganglia neuron by Tkatch and colleagues demonstrating a linear correlation between mean phenotypes and mean genotypes [33]. A quantitative phenotype–genotype correlation at the level of individual neurons was first described for single dopaminergic neurons [46]. However, even quantitative real-time PCR allows only relative or absolute quantification of cDNA molecules, which do not necessarily reflect the number of corresponding mRNA molecules. RT efficiency varies significantly depending on factors such as priming strategies and secondary structure of the individual mRNA transcripts [43,47,48]. Thus, for absolute mRNA quantification, internal RNA standards have to be generated and added to the RT that display similar RT efficiency to the native mRNA in the cytoplasm of single neurons. For single-neuron analysis, internal RNA standards have been designed either by deleting a 48-bp fragment [49] or by inserting a single nucleotide substitution into the target RNA sequence [50]. Even here, the native mRNA target molecules might be transcribed with different efficiency, particularly if they contain complex untranslated regions [48]. In addition, the somatodendritic spatial distribution of the quantified mRNA species might be different, and thus detected differences might be still relative and not absolute [50].

Quantitative genotype–phenotype analysis has in particular facilitated better understanding of the role of Kv3 and Kv4 voltage-gated K^+ channels for fast-firing and slow-firing modes of basal ganglia neurons. In an elegant study, Baranauskas and colleagues [51] have identified the specific single-cell coexpression of Kv3.1 and Kv3.4 mRNA in fast-firing cells, including globus pallidus and subthalamic neurons. They demonstrated that these subunits form heteromeric channels with similar biophysical properties to their native counterparts. These Kv3 channels are essential for fast action potential repolarization, and thus for the fast firing range of the respective basal ganglia neurons. By contrast, Kv4 channels are important to

enable slow firing of other basal ganglia neurons such as cholinergic striatal interneurons [52] and dopaminergic substantia nigra neurons [46]. In dopaminergic neurons, A-type K^+ current densities correlated with spike frequencies as well as with the number of Kv4.3 and KChip3.1 cDNA molecules at the level of individual neurons [46]. Recent studies have investigated the dynamic regulation of Kv4 channel expression: Hahn and co-workers demonstrated that A-type currents, as well as Kv4.3 mRNA, were upregulated in dopaminergic neurons after chronic neuroleptic treatment *in vivo* [53]. Hattori and colleagues showed a correlated increase in Kv4.2 mRNA and A-type current densities during postnatal development of cholinergic interneurons *in vitro* [34]. It will now be very interesting to study how function and expression of these and other ion channels are affected in the adult rodent models of basal ganglia disease.

Transcriptomics of single neurons

To address specific genotype–phenotype correlations with the techniques described here it is mandatory to define a selected set of candidate genes that might be responsible for a given phenotype. A complementary approach is to study a large, or even the complete, set of coexpressed genes in a particular cell type. Microarray-based expression-profiling techniques have recently been successfully adapted for single laser-microdissected neurons [54]. Because current microarray technologies still require micrograms of RNA or DNA for labeling and hybridization, a key factor in making single-cell 'transcriptomics' feasible was development of reliable global amplification techniques. After amplification, the complexity and the relative abundance of transcripts should still reflect the original mRNA composition of the cell. Suitable amplification strategies are based either on advanced versions of the aRNA technique [55,56] or on global PCR protocols [57–59] (Figure 1). Alternative expression profiling techniques such as microSAGE are now also scaled down to the single-cell level [60]. The combination of these techniques will facilitate not only better definition of cell types in the basal ganglia but also identification of concerted changes of gene expression occurring in defined neuronal populations in basal ganglia disease. Data from global brain gene-expression mapping initiatives via automated *in situ* hybridization, bacterial artificial chromosome (BAC)-based transgenes and other techniques [61–63] will provide an increasingly important environment to interpret and complement single-cell expression-profiling data (see the *Nucleic Acids Research* database issue at http://www3.oup.co.uk/nar/database/cat/9).

Future perspectives – from expression snapshots to real-time transcriptional movies

As many of the functional features of basal ganglia neurons can be captured only in the intact network *in vivo*, future studies might aim to combine *in vivo* recording with single-cell labeling and subsequent *ex vivo* harvesting of labeled cells. Novel *in vivo* single-cell gene transfer methods based on electroporation could facilitate this combined approach [64,65]. Also, recent *in vivo* patch-clamping developments might enable direct *in vivo* single-cell harvesting of individual, phenotypically defined neurons [66].

The biggest current limitation is, however, temporal resolution of gene expression, as only snapshots are taken of steady-state gene expression in a single cell. Novel imaging techniques are now beginning to allow the monitoring of single-cell transcriptional activity in real time, which in the future will enable us to watch the complex game played between phenotypic events and transcriptional activity in neurons. For a first flavor of things to come, it is worth watching the first fascinating single-cell transcriptional movies (supplemental movies of Refs [67,68]).

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