A molecular approach to retinal neural networks

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Summary

Our understanding of the computations carried out by neural networks in the central nervous system is limited by our incomplete knowledge of the diversity of cell types and the multiplicity of their functions. In the retina, over fifty cell types encode the spatial, temporal, and chromatic parameters of the incoming light stimuli to generate the messages of action potentials that travel to the brain along the fibers of the optic nerve. We have combined molecular techniques with microscopy and electrophysiology to study a rare cell type in the retina, the dopaminergic amacrine (interplexiform cells). These neurons were labeled in transgenic mice with human placental alkaline phosphatase so that they could be identified in vitro for patch-clamping and analysis of gene expression by single-cell RT-PCR and cDNA array profiling. We have demonstrated that retinal dopaminergic neurons spontaneously generate action potentials in a rhythmic fashion, release dopamine over their entire surface, establish GABAergic synapses and contain proteins whose existence went undetected with more conventional techniques.

KEY WORDS: Dopaminergic neurons, electrophysiology, transgenic mice.

Introduction

The retina is a privileged district of the nervous system because its physical location, the distinctive morphology of its neurons, the regularity of its architecture, and the properties of its input and output favor a unique variety of experimental approaches, which are not possible elsewhere in the brain. If one intends to tackle the problem of the language of the nervous system, the retina seems the ideal focus of interest, since its input can be controlled with great precision and its output is, by and large, known or can be recorded extracellularly with relative ease. For these reasons, the task in hand could seem deceptively simple: if we know how many neurons there are and their connections, chemical mediators and functional interactions, we may be able to discover the detailed mechanisms underlying the receptive field properties of ganglion cells. Thus, a total understanding of the working of a neural network would appear to be within our grasp. Unfortunately, many factors conspire in rendering this goal difficult to attain, especially for the mammalian retina: - There is a large variety of cell types and many of them are too small for a satisfactory electrophysiological analysis in situ. - Each neuronal type probably releases more than a single neurotransmitter. - Quantitative data on the wiring of the various cell types can only be obtained through exhaustive electron microscope work on serial thin sections. - The analysis of ligand- and voltage-gated currents on solitary neurons or retinal slices is limited by the fact that relatively few neuronal types can be unequivocally recognized at the microscope. - A great deal of local processing takes place between small endings, very distant from the cell body and scattered along the length of very fine processes; this activity at the periphery of the neuron may not be accessible to the physiological probes available today. - Extracting functional interpretations from the synaptology may be of limited value, because of the variety of receptors, their complex pharmacology, and the intricacy of the regulatory mechanisms acting upon both transmitter-gated and voltage-gated channels. Adapting a colorful remark by Kupferman (1) to the retina, one can form the impression that retinal neurons swim in a chemical soup. How much information is that soup conveying? How much of it is ignored because of a lack of appropriate receptors? How much of it is involved in subtle adaptive changes of the cells' behavior in response to variations in the ambient illumination? Because of these difficulties, the trend, in the field of mammalian retinal neurobiology, has been heavily biased in favor of morphological studies using the light microscope. Most accomplishments have concerned the analysis of cell populations by using intracellular labeling techniques or immunocytochemistry: an extraordinary number of cell types have been described, as well as their distribution, retinal coverage, contents in conventional transmitters and peptides and uptake of neuroactive substances. This staggering amount of information at light microscope level now needs to be integrated into the retinal wiring, if our ultimate goal is to describe the neural networks that generate the receptive field properties of ganglion cells. From this point of view, much remains to be done: our present information on the wiring of both plexiform layers of the mammalian retina reminds one of a gigantic, incomplete jigsaw puzzle, in which a lot of disconnected details have been reconstructed, but the overall image is still elusive. There are two major reasons for this state of affairs: a) Very few laboratories have studied the quantitative aspects of circuitry in continuous series of thin sections. b) There is only a finite number of simple tricks to label phys-
iologically relevant populations of retinal neurons for the electron microscope; therefore, investigators have to rely ultimately on ultrastructural immunocytochemistry. The difficulties of this approach are well known: with pre-embedding techniques, optimal labeling is only obtained at the surface of blocks or sections and treatments that are necessary to improve the penetration of the reagents have a deleterious effect on the fine structure. With post-embedding techniques, sensitivity is low, because the fixation and embedding procedures decrease the antigenicity of the tissue. From a more general point of view, different antibodies vary in their affinity for the antigen and one needs a whole battery of immunoreagents to label the large number of molecules involved in visual processing.

In contrast with the wealth of morphological and cytochemical information generated by the light microscope, the harvest of physiological data in the mammal is more limited. Most efforts have been devoted to investigating the receptive field properties of ganglion cells. The information, however, that these studies provide on the function of horizontal, bipolar and amacrine cells is necessarily incomplete. Distal to ganglion cells, data have been obtained on horizontal cells and a few types of bipolar and amacrine cells. It has been difficult work, based on endless attempts to penetrate small, delicate cells with electrodes that were either too large to ensure long-lasting recordings or too small to deliver visible tracers. Whole-cell patch clamp recording techniques have been extensively used to investigate the physiological properties of solitary neurons dissociated from mammalian retinas, whereas studies on retinal slices are still relatively rare. It is clear however that, unless the various cell types can be unequivocally recognized on the basis of their morphology or antigenic determinants, the results of these studies cannot be correlated to the cell’s connections in the intact retina. As a result, knowledge of the physiology of cone bipolar cells is largely incomplete, and the precise function of most kinds of amacrine cells remains a mystery.

**Dopaminergic amacrine cells**

Clearly, a radical change of approach was required to increase our chances of success in the study of mammalian retinal networks (2). We wanted to understand which chemical messages are converging upon each type of neuron in the retina, the weight of these messages, their destination at the cell surface and their neuron of origin. Furthermore, if we were able to recognize in vitro, after retinal dissociation, the neuron that represents the target of such messenger molecules, we could correlate its wiring with the cell’s physiology. We would also possess a strategy for designing critical physiological experiments and could investigate the influence of the appropriate constellation of neuroactive substances on the ligand- and voltage-gated currents of the postsynaptic cell. Armed with this combined knowledge of structure and function, if we could identify morphologically the various species of receptors and ion channels expressed by each type of retinal neuron, we could perhaps predict the nature of the neural interactions at sites that are not accessible to physiological experimentation. Ideally, one should be able to use genetic techniques to label the entire population of retinal cells that share a specific protein constituent with a single marker for light microscopy, electron microscopy and electrophysiology. If the cells are labeled, we can count their numbers in the whole retina, study the distribution of their processes in the plexiform layers and map their synaptic connections. We can identify them in vitro after dissociation of the retina and study their ligand- and voltage-gated currents using the patch-clamp technique. Armed with the knowledge of their pharmacology, we can localize their receptors in the intact retina by ultrastructural immunocytochemistry and thus deduce the physiological action of the neurons that are presynaptic to the labeled cells. As a marker, we chose PLAP, whose gene was used for lineage analysis in the retina by Fields-Berry et al. (3). We were planning to link the PLAP gene to regulatory sequences present upstream from the transcription start site of genes that are expressed in specific populations of retinal neurons. When these transgenies are integrated into the genome of fertilized mouse eggs, their foreign promoters become exposed to the complete spectrum of appropriate regulatory signals during the development of specific cell types. As a result, the reporter gene could be expressed in a tissue- and cell-specific manner (4).

We showed that PLAP is a protein ideally suited as a neuronal marker: it is relatively heat-stable, whereas other cell phosphatases are inactivated by a moderate temperature increase (5). Thus, histochemical methods for detection of PLAP activity became highly specific for the neurons that express the transgene. Because of PLAP localization on the outer aspect of the cell membrane, the purple product of the indolyl phosphate reaction for phosphatases faithfully outlines all the details of the neuronal surface. Its connection to the bilayer by a glycan-inositol phosphate tail (6) makes it mobile in the plane of the membrane; it therefore diffuses throughout the neuronal surface and, as a result, dendritic arbors are stained in their entirety. Furthermore, in transgenic lines in which PLAP was expressed by neurons of the central and peripheral nervous system, we were able to follow axons to their termination in distant regions of the brain or body, and thus study pathways and connections. Using the electron microscope, a lead citrate method was found that enabled us to localize PLAP enzymatic activity on the outer aspect of the cell membrane without obscuring the underlying cytoplasm: thus, synaptic contacts could be identified with the utmost clarity. Because of PLAP’s very high pH optimum (pH 10-10.5) (7), its biological activity is probably minimal in the normal environment of the developing and adult retina. Our observation that PLAP-labeled neurons had the same morphology, numbers, and connections as their counterparts in non-transgenic animals confirmed previous evidence that the presence of the enzyme at the cell surface does not affect neuronal development (5).

As the enzyme is localized on the outer surface of the cells, solitary neurons that express PLAP, obtained by dissociation of the retina, could be identified in the living state by immunocytochemistry. For this purpose, a fluorescent monoclonal antibody was used which binds to PLAP without inactivating the enzyme (8). Thus, voltage- and ligand-gated channels could be studied with the patch-clamp technique. As a neuronal marker, PLAP has the shortcoming that it is not visible in vivo without resorting to immunocytochemistry: thus, cells that express the enzyme cannot be
identified in the intact retina for recordings or intracellular injections. On the other hand, PLAP is uniquely suited for light and electron microscopic analysis of the morphology and connections of the labeled cells. We have now constructed chimeric genes coding for both PLAP and a mutated version of GFP, or green fluorescent protein, a spontaneously fluorescent jellyfish protein (9). Both constructs were expressed in an in vitro system and introduced into the mouse genome. The value of PLAP as a marker for specific populations of neurons depends on the chromosomal localization of the transgene and an adequate expression of the gene product. In our hands, the fragment of the promoter of the tyrosine hydroxylase (TH) gene cloned by Banerjee et al. (10) directed the expression of PLAP to catecholaminergic amacrine cells (4). The accuracy of transgene expression in dopaminergic, interplexiform (type 1) amacrines was striking, because every cell expressing TH-like immunoreactivity also expressed PLAP. Furthermore, the density and total number of large PLAP-positive cells was the same as that reported for TH-like immunoreactive neurons in the mouse. Interestingly, the transgene was expressed in type 2 catecholaminergic amacrines, which in the mouse do not stain with antibodies to TH (11). Since type 2 cells in other mammalian species are weakly stained by anti-TH antibodies, it seems that in this instance the transgenic technology was more sensitive than immunocytochemistry. In addition, type 2 amacrines contained less PLAP than type 1 cells, which suggests a parallelism between the regulation of the expression of the transgene and that of the TH gene.

The usefulness of the transgenic technology in labeling homogeneous populations of neurons is limited by the number of cell type-specific promoters that are presently available. We have observed, however, that a truncated promoter of the gene coding for vasoactive intestinal peptide (VIP), acting as an enhancer trap, directed the expression of PLAP to unexpected, but morphologically homogeneous, populations of central and peripheral neurons. This apparently random but cell type-specific expression of the reporter gene proved very useful in labeling other neuronal populations in the absence of specific promoters. We have thus obtained two lines of mice, one in which a class of alpha-ganglion cells are labeled and the other in which rod bipolars are labeled. Since we generated a mouse line in which the dopaminergic cells are labeled by PLAP, we have analyzed their connections, receptors and ionic currents to identify mechanisms and networks that control dopamine release in the mouse retina. Dopamine has an important modulatory action in the retina: it is released when the vertebrate retina is illuminated and is responsible for many of the events that lead to neural adaptation to light (12,13). In cold-blooded animals, dopamine induces contraction of cones and movement of melanin granules in pigment epithelial cells (14,15). In all vertebrates, it decreases the conductance of the gap junctions between horizontal cells and thus reduces the size of their receptive field (16-18); it potentiates the activity of ionotropic glutamate receptors in both horizontal (19) and bipolar cells (20) and, by acting on the AII rod amacrine cell, modifies both the spontaneous discharge of dopaminergic cells is inhibited in the dark by GABAergic amacrines that receive their input from off-bipolars. In bright light, the GABAergic cells generate action potentials and their firing is modulated by the excitation received from on-bipolars. The action potentials spread throughout the dendritic plexus in the inner plexiform layer and cause dopamine release. Our studies of the GABA receptors of dopaminergic cells raised crucial questions on their subunit composition; to solve this point, we used an RT-PCR assay to identify the transcripts of the various GABA receptors subunits at the single cell level and confirmed their presence with both immunocytochemistry (26) and electrophysiology (27).

We also combined carbon fiber amperometry, patch clamping, immunocytochemistry, and electron microscopy to study dopamine release by the perikarya of the dopaminergic cells (28). Because the perikarya of the dopaminergic cells are not presynaptic, events of dopamine release are necessarily extrasynaptic. Amperometric measurements at 22°C showed that solitary perikarya of DA cells in vitro spontaneously released packets of ~20,000 molecules of dopamine at irregular intervals and low frequency. The release events exhibited all of the hallmarks of exocytosis, because they were evoked only upon entry of extracellular Ca²⁺. The spontaneous release was triggered by the action potentials that dopaminergic cells generate in a rhythmic fashion upon removal of all synaptic influences. Thus, extrasynaptic release is controlled by the physiological activity of the cell. Furthermore, it was abolished by the inhibitory transmitter GABA, which suppresses the pacemaker activity of DA cells. Since dopaminergic cells possess extrasynaptic GABA receptors, this is the first demonstration that the composition of the extrasynaptic fluid modulates directly extrasynaptic transmitter release. This may be a general

Direct evidence can only be obtained by recording from dopaminergic cells either in situ or in vitro, in tissue slices or after dissociation of the retina. This study of voltage- and transmitter-gated channels became possible after we obtained the line of transgenic animals in which dopaminergic cells were labeled by PLAP. In these animals, we were able to study their synaptic connections and investigate their GABA, glycine and AMPA-kainate receptors using the patch-clamp technique (4). The most interesting discovery was that dopaminergic amacrines possess a pacemaker activity (22) and thus fire in vivo action potentials in a slow, rhythmic pattern, a property also found in dopaminergic neurons of the substantia nigra and ventral segmental area of the midbrain (23-25). We have studied the time constellations of voltage-gated channels responsible for this behavior, as well as the effects of some neurotransmitters on the firing pattern, and we have formulated the following hypothesis on the control of dopamine release in the intact retina: the spontaneous discharge of dopaminergic cells is inhibited in the dark by GABAergic amacrines that receive their input from off-bipolars. In bright light, the GABA inhibition is removed, dopaminergic cells generate action potentials and their firing is modulated by the excitation received from on-bipolars. The action potentials spread throughout the dendritic plexus in the inner plexiform layer and cause dopamine release. Our studies of the GABA receptors of dopaminergic cells raised crucial questions on their subunit composition; to solve this point, we used an RT-PCR assay to identify the transcripts of the various GABA receptor subunits at the single cell level and confirmed their presence with both immunocytochemistry (26) and electrophysiology (27).

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mechanism that controls volume transmission in the nervous system. Retinal dopaminergic neurons synapse on the perikaryon of all amacrine cells, the neurons that distribute rod signals to on- and off-cone bipolars. We used triple label immunocytochemistry and confocal microscopy to identify the transmitter molecules released at these synapses and the nature of the postsynaptic receptors (unpublished data). We found that clusters of postsynaptic GABA 

A receptors at the surface of all amacrine cell perikarya are situated in register with presynaptic aggregates of organelles immunoreactive for GABA, the GABA vesicular transporter and the vesicular monoamine transporter-2, contained within the dendrites of the dopaminergic neurons. In contrast, D1 and D2/3 dopamine receptors are uniformly distributed throughout the surface of all amacrine cells. We concluded that, like their synapses, retinal dopaminergic neurons release both GABA and dopamine. GABA acts on ionotropic receptors confined to the postsynaptic membrane, whereas dopamine diffuses to more distant, slower-acting metabotropic receptors. Finally, we developed a new technique, SMART7, to globally amplify mRNAs from single neurons and to synthesize single-cell probes to screen a 20K RIKEN cDNA microarray (29). 763 transcripts were identified in DA neurons and the expression of the most interesting and unexpected among them was confirmed by immunocytochemistry in retinal sections. This knowledge has opened up new directions in the study of the biology of DA neurons and their role in the physiology of the retina.

Concluding remarks

Central to a molecular approach to neural networks is the concept of neuronal cell type, that is a class of neurons that receive a specific synaptic input, establish a specific synaptic output and therefore carry out a specific set of computations. When a cell type expresses a specific protein, it can be labeled with a visible marker by using transgenic technology. With this approach, we could identify the dopaminergic neurons of the retina with the light and electron microscopes, and study their synaptic connections, physiological properties and expression of novel and functionally relevant genes.

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