

Review Article

Usefulness of Fluorescent Proteins: Gfp, Dsred and Fruit Fluorescent Proteins Inidentifying Multisynaptic Neuronal Chains. History and Our Own Experience

Ágnes Csáki¹, Zsolt Boldogkői², Enikő Szabó³, and Katalin Köves^{1*}

¹Department of Anatomy, Histology and Embryology, Semmelweis University, Hungary

²Department of Medical Biology, University of Szeged, Hungary

³Department of Conservative Dentistry, Semmelweis University, Hungary

*Corresponding author

Katalin Köves, Department of Anatomy, Histology and Embryology, Semmelweis University, Tűzoltó u. 58. Budapest, H-1094, Hungary, Tel: 36-1-215-6920; Fax: 36-1-215-6034; Email: koves.katalin@med.semmelweis-univ.hu

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Abstract

Investigation of the neuronal connections have been conducted over a long span of time. Debate between researchers about *contiguity* or *continuity* of the nerve elements resulted in a new era in the research of neuronal pathways. The issue has been resolved when synaptic connections were discovered by the electron microscope. This made it possible to use two types of tract tracing methods. First non-trans-synaptic and later trans-synaptic tracers were applied. The former one is suitable to demonstrate direct neuronal connections; the latter are able to describe multisynaptic neuronal circuits. Development of trans-synaptic neurotropic viruses expressing reporter molecules was a great step in this research. GFP, a natural fluorescent protein was discovered in jellyfish more than 50 years ago by Shimomura. Later it was found that GFP fluorescence was stable, species-independent and could be monitored non-invasively using the techniques of fluorescence microscopy and flow cytometry. Later it was recognized that a gene expression cassette encoding the membrane-bound GFP could be inserted in the neurotropic virus genome and could be used successfully for tracing techniques.

ABBREVIATIONS

ABC: Avidin-Biotin-Peroxidase Complex; Bac: Bacterial Artificial Chromosome; BDA: Biotinylated Dextran Amine; BNST: Bed Nucleus of Stria Terminalis; C. elegans: Caenorhabditis elegans; CMV: Human Cytomegalo Virus; DAB: Diaminobenzidine-Tetrahydrochloride; DiI: 1, 1'-Dioctadecyl-3,3,3',3' Tetramethylindocarbocyanine; DIO: Benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-perchlorate 34215-57-1; Ds RED: Discosoma Red Protein; DY: Diamidino Yellow; FB: Fast Blue; FG: Fluoro Gold; FITC: Fluorescent Isotiocyanate; FR: Dextran Fluoro-Ruby; FRET: Fluorescence Resonance Energy Transfer; GFP: Green Fluorescent Protein; HSV: Herpes Simplex Virus; lacZ gene: Gene of β -galactosidase; m Cherry: Monomeric Mutant of Ds Red; MEMRI: Manganese-Enhanced MRI; MRI: Magnetic Resonance Imaging; PHA-L: Phaseolus Vulgaris-Leucoag-

glutinin; PRV: Pseudorabies Virus; PRV-Ba: Attenuated Strain of PRV; PRV-Ba Dup Lac: Recombinant PRV Strain; RSGFP4: Red-Shifted GFP; TagRFP: Tag Red Fluorescent Protein; tdTomato: Monomeric Mutant of DsRed; UV: Ultraviolet Light

INTRODUCTION

Investigations of the neuronal connections have been conducted over a long span of time. More than hundred years ago researchers, Apáthy [1] and Bethe [2,3], hypothesized that nerve impulses propagate along neurofibrils connected in a continuous network throughout all nerve cells. The neurofibrils form delicate threads running in every direction through the cytoplasm of the nerve cells extending into the axon and dendrites and to the next neuron. On the contrary Ramón y Cajal [4], another leading neuroscientist realized that the neurofibrils

are linear bundles constituting dynamic internal skeleton of the nerve cell and do not enter the next neuron; however, the neurons are in a close connection with each other. He said that each nerve cell is an independent entity and nerve synapses transfer nerve impulses from one cell to another. His observations confirm the hypothesis that the nerve elements possess reciprocal relationships in contiguity but not in continuity [5]. Discovery of synapses between the neurons opened a new era [6,7]. The morphology of synapses was described after invention of the electron microscope [8].

The next milestone in the research of neuronal pathways was the introduction of tract tracing techniques. With the use of tracer molecules that were transported ante- and retrograde directions by the axons of the neurons, the researchers could reveal unexplored connections between neurons. The majority of the tracers can not pass through the synapses. Boldogkői and his co-workers [9] as well as Nassi and his co-workers [10] listed the tracers used for neuroanatomical investigations. One of the earliest tracer techniques was the use of radioactively tagged amino acids such as 3H-leucine and 3H-proline. They were taken up by cell bodies, incorporated into proteins and transported anterogradely to axon terminals where they could be visualized with autoradiography [11-13]. This method induced development in neuroanatomy. The first method that exploited retrograde transportation with great success was horseradish peroxidase [14]. Later cholera [15], tetanus toxin [16], biocytin [17] and neurobiotin [18] were used with success to demonstrate direct one-neuronal connections. Phaseolus vulgaris-leucoagglutinin (PHA-L) [19] and biotinylated dextran amine (high molecular weight) (BDA) [20] were used as anterograde tracers with long survival time of the animals after administration. But there is evidence that so called anterograde tracers such as BDA are also transported in retrograde manner [21-23]. To visualize the above mentioned tracers, several methods were used. For example, BDA can be visualized by ABC immunoperoxidase method and nickel intensification of diaminobenzidine-tetrahydrochloride (DAB). To enhance the intensity of the labeling goat anti-biotin antiserum was used. Then biotinylated anti-goat antiserum and ABC were applied and the sections were placed into nickel-DAB chromogen [24].

Another methodological advance was the use of fluorescent tracers. Inorganic fluorescent molecules Fast Blue (FB), Diamidino yellow (DY) or Fluoro-gold (FG) [25-27] were used as retrograde tracers while dextran Fluoro-Ruby (FR) and carbocyanine dyes, DiI and DiO, were used as ante-retrograde tracers [28,29].

Tjälve and his collaborators [30] were the first to demonstrate in fish and later in rats that radioactive Mn^{2+} could be transported to the brain via the olfactory receptor neurons to the olfactory bulb where the ion traversed synapses and reached the olfactory cortex. Mn^{2+} can access neurons through voltage-gated calcium channels. Based upon this fundamental principle, Mn^{2+} has long been used in biomedical research as an indicator of Ca^{2+} influx in conjunction with fluorescence microscopy. A modern method, the manganese-enhanced MRI (MEMRI) utilizes a combination of these properties of Mn^{2+} to trace neuronal pathways in an MRI-detectable manner [31].

HISTORY OF NEUROTROPIC VIRUS TRACT TRACING TECHNIQUES

In the 1990s many papers were published about the suitability of neurotropic viruses to trace neuronal pathways. Some of the neurotropic viruses are a threat to human health. Simple modifications allow them to be used in controlled experimental circumstances, thus enabling neuroanatomists to trace multi-synaptic connections within and across brain regions in experimental animals. The pseudorabies virus (PRV)-Bartha is an attenuated strain developed as a vaccine [32]. Several experiments using rodent models showed that PRV invasion of the central nervous system occurs in an ordered fashion in which the virus passes through synaptically linked neurons, damages cells then replicates in the cell body [33,34]. First an antibody against the virus protein was used to identify the presence of the virus in the nervous system. Mettenleiter and Rauh [35] described a method to obtain high level expression of the bacterial β -galactosidase enzyme by PRV virus. The gX- β -galactosidase fusion gene was produced and inserted in the PRV genome replacing non-essential PRV genomic regions, such as the thymidine kinase gene and the glycoprotein gI-gene, resulting in inactivation of the target genes. The fusion gene remains stably integrated in the viral genome. It therefore appeared ideal as an insertional and easily identifiable marker and greatly facilitates isolation and purification of PRV mutants. After the construction of mutants expressing functional β -galactosidase, PRV was widely used to study neuronal pathways. The presence of the virus was visualized by β -galactosidase antibody [36,37]. Ba-DupLac, a recombinant PRV strain was used for tracing studies since this virus exhibits more restricted transportation kinetics than the kinetics of PRV-Ba [38]. In fact, utilization of Ba-DupLac allowed reduction of the problem to an all-or-none labeling paradigm. Ho and Mocarski [39] inserted a modified Escherichia coli lacZ gene, placed under the control of herpes simplex virus (HSV) alpha 4 or beta 8 regulatory signals, into the HSV-1 genome disrupting the viral thymidine kinase gene. The detection of β -galactosidase expression in neuronal cells indicates that thymidine kinase-deficient viruses are capable of invading mouse neuronal cells and expressing up to the β class of gene product [10]. It was an important observation that the removal of gE and gI membrane glycoprotein genes, which encode the PRV virulence-enhancing factors [40], eliminates the anterograde spreading of the virus [41].

DISCOVERY OF GFP AND OTHER FLUORESCENT PROTEINS IN NATURE

Osamu Shimomura, who received a Nobel Prize in 2008 [42] for his pioneer research, isolated a bioluminescent protein from a glowing jellyfish (*Aequorea victoria*) that gave off blue light [43]. Further studies revealed that the protein's blue light was absorbed by a second jellyfish protein, which in turn re-emitted green light, later called green fluorescent protein (GFP) [44]. Chalfie [45] realized that expression of GFP could be used to map proteins in the transparent nematode worm, *Caenorhabditis elegans* (*C. elegans*). Later, the GFP gene was expressed in *Escherichia coli*. The bacteria glowed green in ultraviolet (UV) light without the addition of any other factors [46].

GFP has become a versatile reporter for monitoring gene expression and protein localization in a variety of cells and organisms. GFP emits bright green light or blue light when excited with UV light. The chromophore in GFP is intrinsic to the primary structure of the protein, and fluorescence does not require additional gene products, substrates or other factors. GFP fluorescence is stable, species-independent and can be monitored noninvasively using the techniques of fluorescence microscopy [47,48]. The protein appears to undergo an autocatalytic reaction to create the fluorophore [49] in a process involving cyclization of a Tyr 66 amino acid residue. Recently a combinatorial mutagenic strategy was targeted at amino acid 64 through 69, which spans the chromophore of jellyfish GFP, yielding a number of different mutants with red-shifted fluorescence excitation spectra [50]. One of these, RSGFP4, retains the characteristic green emission spectrum, but has a single excitation peak. It was demonstrated by fluorescence microscopy that selective excitation of GFP and RSGFP4 allows for spectral separation of each fluorescent signal, and provides the means to image these signals independently in a mixed population of bacteria or mammalian cells. Tsien and collaborators [51] modified the structure of GFP to produce new variants that shine more strongly and produce different colors, such as cyan, blue and yellow. GFP, along with its mutants and homologs, is widely used as *in vivo* fluorescent marker facilitating biomedical studies [52]. Tramier and his co-workers [53] paired the GFP with mCherry protein. mCherry has been successfully fused to many other proteins and used for quantitative imaging techniques including fluorescence resonance energy transfer (FRET). FRET is a mechanism describing energy transfer between two chromophores (light-sensitive molecules) [54]. The GFP/mCherry pair is more resistant against photobleaching. As mCherry, tdTomato and mStrawberry are also Fruit Fluorescent Proteins, which were developed in Tsien's laboratory? Their pairing to bright monomeric orange and red fluorescent proteins improved photostability [55].

Other naturally fluorescent proteins were later found by Matz and his co-workers [56]. They discovered six GFP-like proteins in fluorescent corals (*Discosoma* sp). These proteins exhibited an unexpected color diversity including a red protein called *Discosoma Red* (DsRED). Tsien [57,58] created stable variants of DsRED that glowed in shades of red, orange and pink – complex biological networks can now be labeled using all the colors of the rainbow. Merzlyak and her co-workers [59] reported a Tag red fluorescent protein (TagRFP), which was characterized by high brightness, complete chromophore maturation, prolonged fluorescence lifetime and high pH-stability.

GFP LABELING IN TRACING OF NEURONAL PATHWAYS

Specifically labeled PRV mutants have been used successfully as transsynaptic circuit tracers for definition of central command neurons in the brain [60]. Availability of these recombinant tracers allows the study of even more complex interactions using differentially labeled PRV mutants, and provides means to monitor viral replication and spread without destruction of the cell. Jöns and Mettenleiter [61] improved the method. They isolated and characterized a PRV mutant expressing an engineered GFP

optimized for expression in cells. The GFP DNA was inserted in the non-essential glycoprotein G (gG) gene of the attenuated PRV strain Bartha. The coding sequence was cloned in frame behind the first seven codons of the gG gene under control of the strong gG promoter. On excitation with blue light, live cells infected with the recombinant PRV B80eGFP exhibited bright fluorescence when examined microscopically using filters for fluorescent isothiocyanate (FITC). In fixed samples detection sensitivity was increased by immunofluorescence using an anti-GFP antibody.

In the last 20 years many results were born in exploring neuronal circuits using GFP and other fluorescent protein expressing viruses. It was also an interesting step to engineer PRV viruses which were not toxic even at late stages of infection and these viruses are suitable to give information not only about the early, but about a late phase of infection. These viruses are called timer PRVs [62]. Two retrograde viruses express two different proteins (GFP and DsRED2) with different kinetics and intracellular distribution. Membrane-targeted green fluorescence appears at the early stage of infection (primary fluorescent protein) while the soluble red reporter is detectable several hours later (secondary fluorescent protein).

Very exciting discovery was the creation of transgenic mice that can express GFP in specific neurohormone expressing systems. GFP can be introduced into animals or other species through transgenic techniques, and maintained in their genome and that of their offspring. Van den Poland Ghosh [63] generated GFP transgenic mice in which only one type of neurons were strongly labeled with a fluorescent molecule. These neurons synthesized these molecules internally, allowing the cells, their dendrites and axons to be identified in both living and fixed central nervous system, in slices and culture. The same neurons exhibited GFP beginning early in development, from one generation to the next, allowing cellular and physiological studies of axonal and dendritic growth, anatomical connections, and synapse formation in identified neurons.

CRFp3.0Cre^{GFP} transgenic mice expressing GFP in the corticotropin releasing hormone (CRF) synthesizing cells were used by Dabrowska and her co-workers [64] to demonstrate the CRF projections of oval nucleus of bed nucleus of stria terminalis (BNST) are using an anterograde tracer rAAV5/EF1a-DIO-mCherry. Nowadays many transgenic mice are available. At the Rockefeller University in the frame of GENSAT Project Tg (Vip-EGFP) JN37Gsat, an enhanced GFP (EGFP) reporter gene, followed by a polyadenylation sequence, was inserted into bacterial artificial chromosome (BAC) clone, RP23-25A8, at the initiating ATG codon of the first coding exon of the Vip gene so that EGFP expression is driven by the regulatory sequences of the BAC gene. The resulting modified BAC (BX1866) was used to generate transgenic mice expressing GFP by their VIP neurons. Prönnke and her co-workers [65] characterized the GABA neurons in the cerebral cortex using VIPcre/tdTomato mice and they revealed layer-specific differences. Chi-Sung Chiu and his co-workers [66] constructed a strain of knock-in mice that expressed the mGAT1-GFP fusion in place of the wild type of GAT1 gene. The pattern of fluorescence in brain slices agreed with previous immunocytochemical observations.

APPLICATIONS OF GFP LABELED VIRUS IN OUR EXPERIMENTS

The hypothalamic paraventricular and perifornical and brainstem gigantocellular neurons function as sympathetic as well as parasympathetic premotor neurons. Ample evidence indicates that the descending autonomic pathways from the hypothalamus and the brainstem originate from the paraventricular nucleus (PVN), the perifornical area (Pf), the rostral ventrolateral medulla (rVLM), the locus ceruleus (LC) and the gigantocellular reticular neurons (Gig). GFP labeled retrograde spreading virus MemGreenPRV-R was used to clarify the hypothalamic and brainstem premotor neurons having a double sympathetic-parasympathetic nature involved in the innervation of the lower gingiva and lip. We published two years ago [67] that in intact rats the injection of MemGreenPRV-R resulted in the labeling of postganglionic sympathetic neurons of the three cervical ganglia, the preganglionic neurons in the intermediolateral cell column (IML) of the upper thoracic spinal cord on the ipsilateral side. Labeling was further observed in premotor neurons of the rVLM, the LC, the Gig, the PVN and the Pf. In sympathectomized rats the labeling disappeared from the cervical ganglia, the IML, the rVLM and the LC; however, it persisted in the PVN, the Pf and the Gig indicating that these structures are involved in both sympathetic and parasympathetic responses of the autonomic regulation. Some hypothalamic premotor neurons synthesizing oxytocin innervate the preganglionic neurons in the spinal cord with the use of MemGreenPRV-R labeling and neurotransmitter and neuropeptide immunohistochemistry we demonstrated that a subpopulation of the PVN neurons synthesizing oxytocin is involved in the autonomic regulation of the mammary gland of lactating rats [68] and of the lower gingiva and lip [69].

NEWLY DISCOVERED CENTRIFUGAL VISUAL SYSTEM: PINEALORETINAL PATHWAY

Recently we have published a pinealoretinal connection in adult hamsters, but not in adult rats [70,71]. MemGreenPRV-R was injected into the vitreous body of the right eye of intact or bilaterally sympathectomized male rats. In intact rats the green fluorescent labeling appeared in the trigeminal and the superior cervical ganglia, the IML, the rVLM, the LC, the PVN and the Pf, but was not observed in the pineal body (PB). In sympathectomized rats the labeling was missing in the cervical ganglion, IML, rVLM and LC. In the PB labeling was not seen in either group. When the eye of intact golden hamsters was injected with MemGreenPRV-R virus labeled neurons appeared in the PB. Injection of an anterograde spreading virus (Ka-VHS-mCherry-A-RV) into the PB of golden hamsters resulted in labeling of the retina on both sides. The above-mentioned data indicate that the pinealoretinal neuronal chain (a centrifugal visual pathway) in golden hamsters is present but in adult rats it does not exist.

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