PERSPECTIVE

Shine bright: considerations on the use of fluorescent substrates in living monoaminergic neurons *in vitro*

The biogenic monoamines dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) are major neuromodulators in the mammalian central nervous system (CNS). DA containing neurons are found in i) the mesolimbic system in which cell bodies in the ventral tegmental area (VTA) project axons into the amygdala, cortex, hippocampus and the nucleus accumbens; and ii) the nigrostriatal system in which cell bodies located in the substantia nigra pars compacta send their axons into the dorsolateral parts of the striatum (Bjorklund and Dunnett, 2007). The central noradrenergic neurons are concentrated in distinct brainstem nuclei with the locus coeruleus (LC) being the most prominent nucleus which projects a diffusely arborizing axonal network to most areas of the CNS (Szabadi, 2013). Serotonergic neurons are located in the raphe nuclei in the brain stem with widespread efferent axonal trajectories with a high number of collateral arborizations into many brain regions such as cortical areas, the hippocampus, the basal ganglia and the spinal cord (Sur et al., 1996a, b). Malfunctions of the three monoaminergic systems are associated with different psychiatric and neurological diseases such as depression, anxiety, chronic pain, sleep disorders, schizophrenia, various aspects of drug abuse, Parkinson's disease and Alzheimer's disease.

In recent years, growing evidence was provided that monoaminergic neurons modulate synaptic plasticity predominantly through non-synaptic transmitter release – so-called volume transmission – rather than *via* synaptic signaling "wired transmission" (Vizi et al., 2010). In the concept of volume transmission, exocytotic release of the neurotransmitters occurs both at somatodendritic areas of the cell as well as along axonal varicosities. Released transmitters diffuse through the fluid-filled extracellular space (ECS), and modulate wired excitatory as well as inhibitory neurotransmission by activation of remote non-synaptic receptors. Removal of the neurotransmitters from the ECS is mediated by selective plasma membrane-bound transporters for DA (DAT), NE (NET) and 5-HT (SERT) back into the neurons (Vizi et al., 2010). Like the exocytotic release sites, the neurotransmitter transporters are localized all over the cell body, dendrites and axons (**Figure 1**). Consequently the efficiency of volume transmission is regulated by the activity of the respective monoamine transporter proteins which define the extracellular concentration of the monoamines and of auto- and hetero-receptors which control the neuronal firing rate (Vizi et al., 2010). These findings imply that high somatodendritic transmitter release dampens axonal transmitter release *via* activation of negatively-coupled somatodendritic autoreceptors. On the other end, high activity or density of somatodendritic transporter proteins diminishes transmitter concentration in the ECS, thus decreasing activation of somatodendritic autoreceptors and thereby elevating axonal transmitter release.

The history of volume transmission goes back into the 1980s, when mapping the location of neurotransmitters and their receptors had led to findings which could not be explained by means of classical synaptic neurotransmission. Indeed, in some brain areas, the combination of autoradiography, immunohistochemistry and electron microscopy had revealed that matches between transmitter release sites and neurotransmitter receptors are exceptions rather than the rule (Vizi et al., 2010). Consequently it was the aim of many researchers to study uptake and release of monoamines in more detail by "looking at neurotransmitters through the microscope". Most of these early studies were performed on fixed tissue or cells. In order to study structural and functional aspects of volume transmission in monoaminergic neurons, however, it would be desirable to visualize monoamine uptake and release in living cells. For many years such studies were not possible for two major reasons: i) the relatively low cell number and diffusely arborizing axonal networks of monoaminergic neurons makes them inaccessible *ex vivo*; ii) selective fluorescent substrates for monoaminergic neurons were not available until recently.

In the following part of this article we will discuss the progress made in the last years with respect to the generation of monoaminergic neurons in cell culture and the establishment of selective fluorescent substrates. At the end we will discuss possible perspectives concerning "looking at neurotransmitters through the microscope in living cells". Due to the limited space of this perspective article and based on our own experience we will focus on representative data obtained with serotonergic neurons.

In the last two decades, an increasing number of publications reported on the targeted differentiation of monoaminergic neurons from neuronal progenitor cells, embryonic stem cells (ESCs) and more recently from human induced pluripotent stem cells (hiPSCs). With respect to noradrenergic and serotonergic neurons it has been shown that both can be differentiated from the neuronal precursor cell line 1C11 (Mouillet-Richard et al., 2000). The differentiation into the two monoaminergic phenotypes is mutually exclusive depending on the protocol used. More recently, we had published a protocol for the rapid and efficient generation of highly homogeneous serotonergic neurons from mouse embryonic stem cells (Lau et al., 2010). According to this protocol about 90% of the resulting neurons exhibited a serotonergic phenotype. In line with the concept of serotonergic volume transmission proteins involved in 5-HT release and re-uptake were evenly co-distributed on neurites and cell bodies. Different protocols for the differentiation of dopaminergic neurons from ESCs have been published. However, very few information is accessible with respect to the generation of noradrenergic neurons from ESCs.

Because malfunctions of monoaminergic systems are involved in many psychiatric and neurologic disorders, access to human monoaminergic neurons obtained from patients and healthy human individuals is needed to study possible underlying molecular mechanisms at the cellular level. One common approach is the reprogramming of somatic cells by transient expression of transcription factors to human induced pluripotent stem cells (hiPSC) which can then be further differentiated to neurons. Using various differentiation protocols the efficient generation of excitatory glutamatergic, inhibitory GABA-ergic, cholinergic and dopaminergic neurons as well as spinal cord motor neurons can be achieved; a selective differentiation of serotonergic or noradrenergic neurons has not been reported yet. An alternative approach is the direct differentiation of somatic cells into neurons by forced expression of defined transcription factors (for review see Velasco et al., 2014).

We have combined the generation of stem-cell derived serotonergic neurons together with the application of different fluorescent substances to study transmitter release and uptake. The essential results are shortly summarized below:

A) In order to visualize the recycling of synaptic vesicles, fluorescent styryl dyes such as FM1-43 or FM4-64 are often used (Hoopmann et al., 2012). These dyes bind to membranes

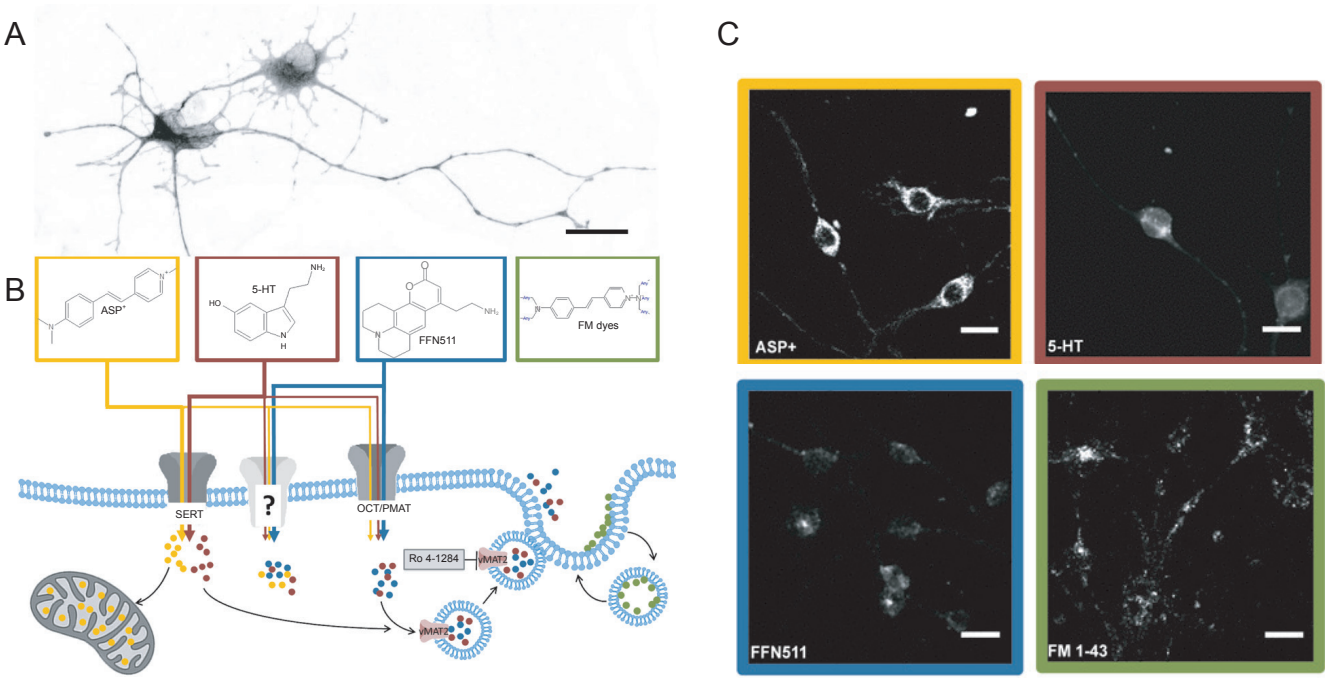

Figure 1 Possible uptake and release mechanisms of fluorescent substrates in serotonergic neurons.
(A) Representative immunostaining against the serotonin transporter in embryonic stem cell-derived serotonergic neurons. In accordance with the concept of volume transmission the serotonin transporter signal is distributed across the whole cell including soma and axons; here pronounced staining is observed at varicosities; bar: 50 µm.
(B) Schematic drawing of the mechanisms of uptake of the fluorescent dyes ASP^+, FFN511, FM and the endogenous substrate 5-HT.
5-HT uptake occurs predominantly *via* the serotonin transporter (SERT) as estimated by incomplete transport inhibition by selective serorotonin re-uptake inhibitors (SSRI). 5-HT at higher concentrations also can enter the cells *via* low affinity, but high capacity transporters like the monoamine organic cation transporter "OCT" and the plasma membrane-bound monoamine transporter "PMAT". Whether there exist additional unspecific transporters "?" that enable 5HT uptake is still under investigation. Inside the cell 5-HT accumulates into vesicles *via* vMAT2. ASP^+ is taken up to large extent by SERT; whether it is transported also by other transporters like OCT/PMAT or yet unidentified transporters "?" is not known. Inside the cell ASP^+ accumulates into mitochondria but not into vesicles. FFN511 does not enter the cells *via* SERT, as its uptake is not blocked by SSRIs; the mechanism how FFN511 is taken up into the serotonergic neurons (shown here) and dopaminergic neurons (Gubernator et al., 2009) is not identified yet; Like 5-HT, FFN511 accumulates in synaptic vesicles *via* vMAT2. FM styryl dyes bind to the cells membrane and can be endo- and exocytosed.
(C) Representative stainings for: FM1-43: 5 min at 2 µM; ASP^+: 30 sec at 10 µM; FFN511: 5 min at 10 µM, and 5-HT: 10 min at 500 µM. Images FM1-43, ASP^+ and FFN511 are Z-projections of confocal stacks. In accordance with the concept of volume transmission the fluorescent signals of ASP^+ as well as vesicular stainings (FM1-43, FFN511 and 5 HT) are observed in cell soma and on all neurites. Bars: 20 µm.
5-HT: 5-Hydroxytryptamine; ASP^+: 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide; FFN511: fluorescent false neurotransmitter 511; vMAT2: vesicular monoamine transporter 2.

whereupon they exhibit a higher fluorescence than in aqueous solution. After stimulation of neurons *e.g.*, by application of 60 mM KCl in the presence of FM dyes, re-endocytosed vesicles are filled with the dyes and fluorescent spots light up under the microscope. After recovery of the cells, a second pulse of 60 mM KCl will induce new exocytosis and thus vesicle fusion with the membrane and release of the dyes ("destaining"). This method can be applied to all kinds of excitable cells and we have used it to visualize depolarization-induced somatodendritic exocytotic events in stem cell-derived serotonergic neurons (Lau et al., 2010).

B) As a more selective substrate for monoaminergic neurons, the fluorescent compound ASP^+ (4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide) has been developed (Mason et al., 2005). This fluorescent analog of the neurotoxin MPP^+ is taken up by DAT, NET and SERT into monoaminergic neurons. Studies on ASP^+ uptake into stem cell-derived serotonergic neurons revealed that the selective serotonin re-uptake inhibitor (SSRI) citalopram did greatly, but not completely, diminish ASP^+ uptake. Inside the cell, ASP^+ accumulation was not blocked by inhibition of the vesicular monoamine transporter vMAT2. These findings suggest that in serotonergic neurons, ASP^+ is partly taken up by SERT, however, is not imported into synaptic vesicles (Lau et al., 2015).

C) Very recently a new fluorescent monoamine analog (Fluorescent False Neurotransmitter, FFN511) has been synthesized which allowed to visualize dopamine release from individual presynaptic terminals in live cortical-striatal acute slices (Gubernator et al., 2009). Here FFN511 diffused in a DAT independent way into dopaminergic neurons where it accumulated into synaptic vesicle as revealed by effective vMAT2 blockade; comparably we had observed SERT-independent uptake and vMAT2-driven transport of FFN511 into synaptic vesicles in serotonergic neurons (Lau et al., 2015). The mechanism how FFN511 enters monoaminergic neurons has not been identified yet.

D) 5-HT is known to emit fluorescence upon excitation at 320–460 nm and can be visualized and quantified in stem cell-derived serotonergic neurons. Here, 5-HT uptake occurred through SERT and a yet unknown mechanism as revealed by incomplete uptake inhibition by citalopram; inside the cell 5-HT accumulation into vesicles was blocked by the vMAT2 inhibitor Ro 4-1284 (Lau et al., 2015).

A summary of the characteristics of the different fluorescent

substrates described above is provided in **Figure 1B** and representative micrographs of the findings are shown in **Figure 1C**. In accordance with the concept of volume transmission all fluorescent signals of transmitter uptake (SERT immunofluorescence in **Figure 1A**) and ASP^+ as well as vesicular stainings (FM1-43, FFN511 and 5-HT; **Figure 1C**) are observed in the cell soma and on all neurites.

The diversity of these fluorescent substrates with different properties combined with the possibility to differentiate monoaminergic neurons from different sources opens new perspectives to study the: (1) Activity and/or density of cell surface-located monoamine transporters that control the concentration of bioactive extracellular neurotransmitters, and (2) effects of substances which impact monoamine release in living monoaminergic neurons *in vitro*.

For the first aspect, ASP^+ would be the tool of choice since it is in large part transported by DAT, NET and SERT. Hereby ASP+ uptake correlates to the amount of cell surface expressed transporter proteins. Therefore this dye is well suited to study molecular mechanisms in more detail how drugs influence transporter activity and or cell surface. Importantly, ASP^+ fluorescence is photo-stable and yields good signal to noise ratios for quantitative image acquisition. Since ASP^+ is accumulated into mitochondria and not into vesicles, it cannot be used to study neurotransmitter release (Mason et al., 2005).

In contrast to ASP^+, the "false fluorescent neurotransmitter" FFN511 is transported by vMAT2 into monoaminergic vesicles (shown for DA neurons (Gubernator et al., 2009) and 5-HT neurons (Lau et al., 2015)) and thus can be used to visualize synaptic vesicles. Upon depolarization-induced exocytosis the vesicles release their content which goes along with a significant reduction of FFN511 stained particles. The finding that loading as well as depolarization-induced destaining can easily be quantified by laser scanning confocal microscopy renders FFN511 a well suited selective fluorescent substrate to perform investigations on structural and functional aspects on the anatomical distribution of both axonal and somatodendritic monoamine release sites. For example, targeted application of selective agonists of excitatory neurotransmitter receptors allows to functionally narrowing down the cellular localization of these receptors by identifying induced monoamine release sites. On the other hand, general induced depolarization (*e.g.*, by 60 mM KCl) in the presence of selective agonists of inhibitory transmitter receptors will allow to precisely determine their cellular localization by visualization of distinct sites with diminished FFN511 release. Here, the more selective characteristics of FFN511 provide an advantage over the FM styryl dyes as it is taken up into vesicles without the need of prior exocytotic events. Therefore FFN511 labels vesicles primed for neurotransmission and assigned to reserve pools.

It is a special feature of serotonergic neurons that the fluorescence of the natural transmitter, 5-HT, can be directly visualized under the microscope. However, this is only possible at higher concentrations (> 200 μM). At these concentrations, 5-HT is for the most part (but not completely, see **Figure 1**) taken up by SERT and subsequently accumulated into vesicles. Thus, in serotonergic neurons, 5-HT itself at higher concentrations can be employed to study both, neurotransmitter uptake and release, under the microscope.

In summary, depending on the experimental approach the newer techniques in generating defined neuronal populations *in vitro* together with the development of selective fluorescent substrates for monoaminergic neurons enable detailed investigations on structural and functional aspects of non-synaptic monoamine uptake and release in living neurons. Moreover, the possibility to selectively differentiate neurons from hiPSCs from genotyped and phenotyped individuals opens the possibility to compare the actions of drugs and drugs of abuse on monoaminergic neurotransmission in neurons from patients and from healthy subjects.

Finally, the development of specific fluorescent substrates for cholinergic and glutamatergic neurons will enable the quantification of neurotransmitter uptake and release also in living excitatory neurons. Such an approach surely would be an additional valuable tool to estimate the viability of neurons also during neuroregeneration.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB636) to PS and FM.

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***Accepted:** 2015-06-15*
***doi:** 10.4103/1673-5374.165223* ***http**://www.nrronline.org/*
Schloss P, Matthäus F, Lau T (2015) Shine bright: considerations on the use of fluorescent substrates in living monoaminergic neurons in vitro. Neural Regen Res 10(9):1383-1385.

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