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Abstract

Intracellular signaling with cyclic nucleotides are ubiquitous signaling pathways, yet the dynamics of these signals profoundly differ in different cell types. Biosensor imaging experiments, by providing direct measurements in intact cellular environment, reveal which receptors are activated by neuromodulators and how the coincidence of different neuromodulators is integrated at various levels in the signaling cascade. Phosphodiesterases appear as one important determinant of cross-talk between different signaling pathways. Finally, analysis of signal dynamics reveal that striatal medium-sized spiny neuron obeys a different logic than other brain regions such as cortex, probably in relation with the function of this brain region which efficiently detects transient dopamine.

Introduction

Neuromodulatory processes are involved in the regulation of a vast number of neuronal properties in the brain, and a good part of their effect is mediated through cyclic nucleotide second messengers. For example, monoaminergic transmitters (dopamine, noradrenaline, serotonin), acetylcholine, and a wide palette of neuropeptides, play important functional roles through the activation of various selective receptors, which ultimately exert their effect through intracellular second messengers (Björklund and Dunnett, 2007; Iversen and Iversen, 2007; O'Donnell et al., 2012; van den Pol, 2012). This non exhaustive review thus focuses on some recent advances in the understanding of the cAMP/PKA and cGMP/PKG signaling pathways in vertebrate neurons. Genetically-encoded FRET biosensors now provide large signals that are fairly easy to record, and we will briefly describe the ratiometric approach to monitor them. By providing measurements with sub-cellular resolution in primary neuronal cultures, brain slices and even in vivo, this approach is literally changing our point of view on pharmacology. While a number of GPCR and downstream signaling enzymes have been studies in heterologous systems, it is now possible to measure the effect of the natural agonist or pharmacological agents in situ, sometimes revealing discrepancies with former approaches. Moreover, since many neuromodulators interact in vivo, biosensor imaging can reveal how different signaling pathways interact, in particular through the regulation of phosphodiesterase (PDE) activities. Timing is an important aspect of neurophysiology, and biosensors provide a high temporal resolution to measure transient events. Besides, all these studies provide a vast amount of data in spatial and temporal dimensions, which are priceless for the development of numerical models of intracellular signal integration.

Methodology

Historically, conformational changes in proteins were detected by labeling specific amino-acids with fluorophores and then measuring FRET changes between these two fluorophores as an indicator of a change in distance and/or orientation. FRET is a physical process whereby the energy of an excited fluorophore (the donor) is transferred by resonance to a neighboring fluorophore (the acceptor), a phenomenon that strongly depends on the relative distance and/or orientation between the donor and acceptor (Wouters et al., 2001; Jares-Erijman and Jovin, 2003; Padilla-Parra and Tramier, 2012). The Förster distance is the distance between the donor and acceptor at which 50 % of FRET efficiency occurs, generally ranges between 2 and 8 nm. This principle has been applied to create the first sensor for cAMP, FlCRhR, using recombinant PKA labelled with fluorescein and rhodamine, and detecting cAMP-induced PKA dissociation by a loss of FRET (Adams et al., 1991). Multicolor variants of GFP allowed for the development of biosensors encoded by a single gene and constituted of a sensor domain sandwiched between two fluorophores. Such biosensors exhibit a change in FRET efficacy when the biological signal of interest changes the conformation of the sensor domain (Zhang et al., 2002; Tsien, 2010).

Measurement methods

There are different methodological approaches to measure FRET between a donor and an acceptor, of which only two are reliable enough to be routinely used for live imaging of

biosensor signals in living cells: intensity-based ratiometry and fluorescence life-time imaging microscopy (FLIM). While FLIM imaging has it own merits that have been largely described (Padilla-Parra and Tramier, 2012; Chen et al., 2014, 2017; Tang and Yasuda, 2017; Ma et al., 2018), including in this issue (note to the editor: please refer to Haining Zhong's review in the same issue), the ratiometric approach is somewhat underrated, in particular considering that this method is very simple and cheap to implement, and nonetheless provides low-noise quantitative measurements. The biological signal of interest induces a conformational change of the sensor domain, which leads to a change in FRET efficacy between the donor and acceptor fluorophores. This change in FRET efficacy goes from one FRET level, characteristic of the sensor in "off" state, to another FRET level, characteristic of the sensor in the "on" state. The FRET change can be an increase, such as with AKAR-type biosensors when phosphorylated by PKA, or a decrease, like Epac based sensors binding cAMP. Although a measurement of absolute FRET change is interesting for theoretical understanding of biosensor conformational changes, a precise quantification of FRET level is useless in routine live cell measurements: it is sufficient to determine the relative fraction of biosensors in the on and off states. This equilibrium level can be directly derived from the ratio of acceptor (IA) and donor (ID) intensities. In the case of a biosensor in which activation increases FRET, the ratio IA / ID conveniently reports biosensor activation as an increase in ratio measurement. If biosensor activation decreases FRET, the ratio ID / IA reports biosensor activation. In both situations, the measured ratio evolves between the ratio for the biosensor in the off state (Rmin), and the ratio when all the biosensor is in the on state (Rmax).

An important strength of the ratio calculation is the cancellation of various experimental artifacts through the calculation of a ratio. Experimental biases such as changes in biosensor concentration, cell movement, focus drift. are canceled through the ratio calculation, as long as IA and ID are affected in a same linear way. It is important to check whether this main advantage of ratiometric calculation might get lost when corrections such as bleedthrough subtraction or correction for direct excitation of the acceptor are applied to either donor or acceptor intensities. While such correction makes the maximal ratio change appear larger in amplitude, it may be at the expense of losing the aforementioned advantages of ratiometric correction. Moreover, the added calculation step actually increases the noise in the measurement (Ducros et al., 2009).

Collecting signal

Ratiometric data are easy to collect with a fluorescence microscope equipped with a single excitation wavelength for the donor, and a device like a filter wheel or an emission splitter to measure fluorescence emission at the wavelengths of donor and acceptor. Wide-field epifluorescence is commonly used, providing minimal donor excitation - and therefore minimal bleaching -, and allowing for the measurement of a large number of data points (up to several hundred). Even though wide-field fluorescence imaging does not provide optical sectioning, it nevertheless appeared quite easy to monitor individual neurons in brain slice preparations, as long as cell bodies are at a sufficient distance. When unambiguous isolation of individual neurons is required, 3D resolution can be obtained with two-photon microscopy with dual emission (Yapo et al., 2017; Nair et al., 2019). Optical fibers can even be used to ratiometrically monitor a biosensor signal in deep brain regions in vivo (Jones-Tabah et al., 2020).

Signal interpretation

Calculating cAMP concentration in brain slices.

The ratiometric measurement is intrinsically quantitative, following principles initially established for chemical calcium dyes like fura-2 (Grynkiewicz et al., 1985; Tsien, 1999), and more recently adapted to cyclic nucleotides (Violin et al., 2008; Mironov et al., 2009; Börner et al., 2011; Russwurm and Koesling, 2018). This calibration uses the EC50 and Hill coefficients, determined in vitro for most published cAMP sensors. In addition, the conversion of ratio measurements into cAMP concentrations requires the determination of Rmin and Rmax, which are the ratio values in the absence and in the presence of saturating concentrations of cAMP, respectively. Rmax is obtained by maximally stimulating ACs while inhibiting phosphodiesterases or using a membrane permeant cAMP analogue like 8-Br-2′-O-Me-cAMP-AM. As a routine, we terminate all our experiments with an application of forskolin and IBMX to reach biosensor saturation and determine the Rmax value for each cell. It is more difficult to determine Rmin in every experiment and instead, the average basal cAMP level in a typical set of experiment should be determined: ACs can be inhibited with dideoxy-adenosine, SQ22536 or MDL-12,330A (Mironov et al., 2009; Börner et al., 2011; Castro et al., 2013; Polito et al., 2015), and the hypothesis is made that such inhibition is sufficient for the biosensor to reach Rmin. In striatal spiny neurons, SQ22536 (200 μM) slightly decreased the basal ratio of the Epac-S^{H150} biosensor, indicating a basal cAMP concentration in the range of 100 nM (Mota et al., 2021). Knowing Rmin, Rmax, EC50 and the Hill coefficient, the ratio value can be converted into an estimate of cAMP concentration (Russwurm and Koesling, 2018).

The conversion of ratio to concentration tends to artificially blow up the noise in traces showing calculated cAMP concentrations when the ratio gets close to Rmin and Rmax. Instead of showing traces converted in cAMP concentration on a log scale, which typically enhances these noises, we rather display and perform statistics on ratio values, and add an axis showing the estimated concentration besides the ratio trace (Mota et al., 2021).

Biosensor sensitivity is indeed a key determinant for the detection of a response and recent sensors considerably improved their dynamic range and signal-to-noise ratio. For example, the first generation of cAMP sensor, Epac1-camps, reported no change in response to several monoamine and neuropeptide neuromodulators (Castro et al., 2010; Hu et al., 2011; Castro et al., 2013). Improved sensors such as ${}^{T}Epac^{VV}$ (Klarenbeek et al., 2011), Epac-S^{H150} (Polito et al., 2013) and Epac-S^{H187} (Klarenbeek et al., 2015) with a much larger maximal ratio change and a fairly extended detection range now allow cAMP measurements over a much broader range of concentration. For example, the β-adrenergic response in pyramidal cortical neurons was much better resolved with newer Epac-S^{H150} (Castro et al., 2010; Luczak et al., 2017). Modest cAMP signal are nonetheless sufficient to activate PKA and trigger cytosolic and nuclear responses of AKAR-type sensors, which thus appear as the most sensitive detectors for this pathway (Castro et al., 2010; Hu et al., 2011; Nomura et al., 2014).

Various biosensors for cGMP have also been developed, which have been reviewed in great details (Russwurm and Koesling, 2018). Two more recent biosensors for cGMP have been described: PfPKG, with a high sensitivity to cGMP (EC50 of ~20 nM) (Calamera et al., 2019),

and cyGNAL, with less sensitivity to cGMP (EC50 of ~0.5 µM) but better selectivity against cAMP (Betolngar et al., 2019).

Buffering effect

Biosensor concentration may complicate the interpretation of measurements: if the biosensor is present in large excess compared to cAMP, only a small fraction of the biosensor will switch to the on state, and the ratio will report a lower cAMP concentration than what would have occurred if the biosensor were at a lower concentration. In parallel, excessive biosensor concentration, by buffering cAMP, may prevent cAMP action on its physiological targets. Biosensor concentration is usually in the low micromolar range, a concentration that should not affect the biological signal. However, biosensor concentrations of several tens of micromolar can be reached easily (van der Wal et al., 2001; Drobac et al., 2010), where it certainly dampens the cAMP signal. While determining accurately the concentration of a biosensor inside a living cell is technically delicate, it is quite easy to verify the impact of biosensor concentration on the cAMP responses by analyzing the relationship between the amplitude of the cAMP measurement of interest and the biosensor fluorescence level. If such buffering effect were involved, neurons showing a low fluorescence intensity, indicative of low biosensor expression level, should display responses of larger amplitude than neurons showing high fluorescence intensities. Such negative trend was not observed in pyramidal cortical neurons showing that a buffering effect did not affect the responses in these conditions (Castro et al., 2013). However, the buffering effect was clearly observed in vascular smooth muscle cells, with a statistically lower cAMP response in the cells expressing the biosensor at the highest level (Figure 5 in (Vallin et al., 2018)). Simulations using a model built from our biosensor recordings showed that this buffering effect is negligible in striatal medium-sized spiny neurons, in relation with the high turn-over of cAMP in this neuronal type (Yapo et al., 2017).

Activation of cyclic nucleotide signaling in situ

Cellular environment plays an important role in receptor function and signal integration. Thus, the choice of differentiated neurons in their physiological context, i.e., in brain slice preparations, is of great importance. It provides morphologically intact neurons, which maintain their functional network connections, as well as endogenous receptor expression.

Functional receptors in specific neuronal types in situ

Various GPCR in pyramidal cortical neurons

Biosensor imaging are ideal tools to reveal *in situ* which G-protein coupled receptors (GPCR) are involved in a neuromodulator's action. Noradrenaline or adrenaline are released in different brain areas where it modulates the activity of neural circuits and enhances synaptic plasticity, by affecting neuronal and non-neuronal cells (O'Donnell et al., 2012). These transmitters activate, among others, β-adrenergic receptors (β-AR). Mouse pyramidal cortical neurons express both β1-AR and β2-AR. Selective pharmacology demonstrated that noradrenaline action on cAMP/PKA pathway selectively involves β1-AR (Castro et al., 2010). Photostimulation of channelrhodopsins in locus coeruleus fibers in the neocortex triggers the release of noradrenaline. This release transiently increases the PKA response in somata and

dendrites of individual pyramidal cells, and this effect is dampened by the co-activation of α2 adrenoceptors and by noradrenaline re-uptake (Nomura et al., 2014; Ma et al., 2018; Nomura et al., 2020). Functional dopamine D1-like receptors have also been revealed by AKAR or Epac imaging in pyramidal cortical neurons (Castro et al., 2013; Yapo et al., 2018).

A number of neuropeptides are expressed in the brain, each of them accompanied by a group of cognate GPCR. These neuropeptides are involved in a number of physiological functions, from the control of homeostasis to cognition (van den Pol, 2012). Pyramidal cortical neurons express VPAC1 and PAC1 receptors, both of which can be activated by the local release of the neuropeptide Vasoactive Intestinal Peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP). These neuropeptides are involved in synaptic transmission and neuronal plasticity, with interesting therapeutic potential to treat cognitive defects (Ciranna and Costa, 2019; Cunha-Reis and Caulino-Rocha, 2020). Using AKAR2 biosensor and pharmacological receptor modulation, Hu *et al.,* (2011) demonstrated that VIP and PACAP action are mostly mediated in neocortical neurons by VPAC1 receptor and PAC1 receptor, respectively (Hu et al., 2011). AKAR measurements also revealed important differences in the efficacy of these neuropeptides, in terms of response amplitude and time-course.

Dopamine receptors in the striatum

Dopamine plays a critical functional role in the striatum, where it binds to D1 or D2 receptors, expressed on two distinct sub-populations of medium-sized spiny neurons (MSNs). It is widely accepted that D2-expressing MSNs respond to low dopamine concentrations, resulting from the tonic activity of dopaminergic inputs, whereas D1-expressing MSNs respond to the high dopamine concentrations resulting from phasic activity in dopaminergic inputs. This notion derived from binding measurements or analyzing dopamine receptors in heterologous systems, which showed, in these conditions, that D2 receptors had a much higher affinity for dopamine than D1 receptors. This static view must be revised with novel evidences indicating that D2 receptors in vivo can exist in low and high affinity states (Skinbjerg et al., 2012). This question could be addressed with biosensor imaging in striatal brain slices, where the effect of dopamine on D1 and D2 receptors could be measured simultaneously on the two distinct sub-populations of MSNs. Caged-dopamine (NPEC-DA) was used to deliver dopamine for a short and specific time mimicking transient dopamine. EC_{50} displayed similar value for D1 and D2 MSN in the range of sub-micromolar, differing only by a factor of two (Yapo et al., 2017), a much smaller difference than commonly assumed, but consistent with early biochemical measurements (Enjalbert and Bockaert, 1983; Weiss et al., 1985). Modeling of the cAMP/PKA cascade suggest that, independently of low receptor affinity, D2-expressing MSNs could respond to an interruption in the tonic release of dopamine (Yapo et al., 2017).

This novel view on dopamine receptor sensitivity was confirmed in an interesting transgenic mouse model expressing $^{T}Epac^{VV}$ developed recently: primary neuronal cultures from these animals showed a sensitivity to dopamine that was even higher in D1 MSNs than in D2 MSNs (Muntean et al., 2018). This work also analyzed adenosine and opiod responses, and imaged in brain slices cAMP responses to endogenous dopamine released by optogenetic stimulation of dopaminergic neurons (Muntean et al., 2018).

In Parkinson’s disease, the degenerescence of dopaminergic neurons in the substantia nigra deprives the striatum from its physiological dopamine input. This lack of dopamine on D1 MSNs induces an hypersensitivity through a more efficient D1R-Golf coupling (Corvol et al., 2004). Biosensor imaging showed these hypersensitive responses in brain slices (Mariani et al., 2019) and in vivo (Jones-Tabah et al., 2020).

Monitoring the activation of the cGMP signaling pathway

NO-mediated activation of guanylyl cyclase pathway plays an important role in neurobiology, in synaptic plasticity and in the regulation of glutamate release (Kleppisch and Feil, 2009; Garthwaite, 2019). For example, cerebellar LTD requires glutamate-induce calcium signals, leading to the release of NO and activation of PKG (Daniel et al., 1998). Such signalling cascade was explored in great details using a transgenic mouse line expressing the cGi-500 sensor (Giesen et al., 2020). Using primary cultures and hippocampal and cortical brain slices from this mouse line, the authors characterized the successive steps: a glutamate signal, acting on NMDA and AMPA receptor, triggers a calcium influx through L-type calcium channels which leads to NO production by nNOS enzyme, eventually increasing cGMP.

Another source of cGMP production is the activation of natriuretic peptide receptors. The recent development of highly sensitive cGMP biosensors of the PfPKG series reported cGMP signals in response to brain natriuretic peptid (BNP) in cardiac stellate ganglia (Liu et al., 2018; Calamera et al., 2019).

Shaping the cyclic nucleotide signal

Receptors crosstalk at the level of adenylyl cyclases

Biosensor imaging opens up a novel way to analyze the competing or synergistic interactions between different extracellular signals. Indeed, neurons express a wide diversity of GPCR, some of them coupled to cAMP signaling, either positively or negatively. While the main coupling is generally well documented for each GPCR, it is much more difficult to predict the outcome of simultaneous activation of GPCR coupled in an opposite way, as is occurs in physiological situation.

The sub-population of striatal medium spiny neurons which express Gi-coupled D2 dopamine receptors also express the Golf-coupled adenosine A2A receptor. Both dopamine and adenosine are present simultaneously in the striatum, with large variations of dopamine levels depending on the tonic or phasic activity of dopaminergic inputs. In the continuous presence of adenosine, biosensor imaging indeed shows that D2 receptors efficiently switches off cAMP production in spiny neurons, leading to PKA de-activation (Yapo et al., 2017; Mota et al., 2021).

Symmetrically, the other sub-population of striatal medium-sized spiny neurons express the Golf coupled D1 receptor together with the Gi/o-coupled M4 muscarinic receptor. Although rare in number, cholinergic interneurons in the striatum cover a wide territory with their axonal arborization and play an important functional role (Gonzales and Smith, 2015). While these neurons release acetylcholine tonically, a phasic dopamine signal stops their activity (Aosaki et al., 2010). Theoretical work suggested that this transient lack of acetylcholine might be required

to let a positive cAMP signal develop in response to D1 receptor stimulation (Nair et al., 2015). Biosensor imaging confirmed the high affinity of M4 muscarinic receptors and their efficacy in shutting-off cAMP production (Figure 1). In vivo, this effect of M4 was sufficient to reverse increased locomotion activity in a mouse model of hyperactivity (Nair et al., 2019).

It is interesting to note that MSNs mainly express type 5 adenylyl cyclase, which is specific of this brain region (Matsuoka et al., 1997). Recent molecular modeling work revealed that type 5 adenylyl cyclase can bind both Gs and Gi/o simultaneously, switching the catalytic site into the inactive conformation (van Keulen et al., 2019). Whether other adenylyl cyclases in other brain regions also give preeminence to Gi/o remains to be determined.

Another signaling situation involving complex receptor to cyclase coupling was addressed with biosensor imaging: the authors show that activation of M1 muscarinic receptors, in spite of being coupled to Gq proteins, leads to an activation of an AKAR-type biosensors (Chen et al., 2017). The precise mechanism, which remains to be identified, probably involves calcium and PKC. This work highlights the complexity of cross-talks between signaling pathways and the importance of direct measurements at different levels of a signaling cascade in the native environment.

Phosphodiesterase activities determine the profile of cyclic nucleotide signals

The cAMP signal results from a constant equilibrium between cAMP production by adenylyl cyclases and cAMP degradation mainly by phosphodiesterases (PDEs), which hydrolyze cAMP and/or cGMP into the AMP and/or GMP. More than 50 PDE isoforms, encoded by 21 genes have been described so far (Keravis and Lugnier, 2012). In the last decade, the development of a large palette of specific PDE inhibitors allowed for a better understanding of specific phosphodiesterase functions in cAMP and/or cGMP signaling in various cell types (Maurice et al., 2014; Baillie et al., 2019), in parallel with the development of a number of novel drug development programs (Menniti et al., 2006; Baillie et al., 2019). Indeed, multiple PDE isoforms are expressed in a same cell, with specific regulatory domains, enzymatic properties and multiple intracellular localizations. Their affinity for different substrate concentrations as well as modulation by other intracellular signals, places them at the crossroad between different signaling pathways, allowing for a finely tuned interplay between different neuromodulatory signals.

PDE10A: a major regulator of cAMP/PKA signals in the striatum

The complexity of the regulation exerted by PDEs can be observed in striatal neurons, which express high levels of PDE1B, PDE2A and PDE10A, and moderate levels of PDE4B (Seeger et al., 2003; Coskran et al., 2006; Heiman et al., 2008; Lakics et al., 2010; Kelly et al., 2014). The PDE10A protein is present almost exclusively and at very high levels in both D1 and D2 MSNs (Seeger et al., 2003; Coskran et al., 2006; Lakics et al., 2010). PDE10A displays high (sub-micromolar) affinity for cAMP (Wang et al., 2007; Poppe et al., 2008) and biosensor imaging highlights PDE10A as the main regulator of tonic AMP production by adenylyl cyclases (Polito et al., 2015).

In addition, PDE10A shows a prominent functional role in the regulation of the cAMP/PKA response in both D1 and D2 MSNs (Mota et al., 2021), as shown by cAMP measurements in striatal brain slices. This work showed that PDE10A is required to degrade cAMP to a sufficiently low level to allow for PKA de-activation: when PDE10A is blocked, dopamine signals can no longer be detected at the level of PKA. Previous studies showed differences between D1 and D2 MSNs in their responsiveness to PDE10A inhibitors, both in vivo and in brain slices (Threlfell et al., 2009; Polito et al., 2015). This D2/D1 imbalance was consistent with PDE10A inhibitors mimicking the D2 antagonistic action of antipsychotic agents, which had sparked considerable interest in PDE10A as a potential therapeutic target to treat schizophrenia (Kehler and Nielsen, 2011; Schülke and Brandon, 2017; Harada et al., 2020). So far, this hope has not met clinical success (Menniti et al., 2020), possibly because inhibiting such an essential signaling enzyme profoundly destabilizes signal integration in MSNs (Mota et al., 2021). Compared to PDE10A, PDE2A and PDE4 display much lower affinity for cAMP and therefore preferentially regulate high cAMP concentration. Although not critically required, modulation of these PDEs may be of therapeutic interest, since they determine the maximal amplitude as well as steady-state cAMP levels that can be obtained in striatal neurons with dopamine or adenosine (Mota et al., 2021).

PDE2A: Mediator of the NO/cGMP signaling

PDEs can mediate interactions between different signaling pathways. For example, LTS interneurons in the striatum express high levels of neuronal nitric oxide synthase (Tepper et al., 2018), suggesting that NO may play a role in the regulation of dopamine action. Indeed, NO diffuses throughout the striatum and activates cGMP production by soluble guanylyl cyclases (GCs), which is highly expressed in the MSNs (Ariano et al., 1982; Matsuoka et al., 1992; Ding et al., 2004). Biosensor imaging clearly confirmed the powerful activating action of NO donors on cGMP production in various brain regions (Hepp et al., 2007; Betolngar et al., 2019; Giesen et al., 2020). This cGMP signal can activate PDE2A, which specific feature is its allosteric activation by cGMP binding to a regulatory domain. cAMP/PKA imaging showed that this NO/cGMP/PDE2A cascade indeed reduced cAMP and PKA responses triggered by transient dopamine in striatal neurons (Polito et al., 2013). A similar observation was made in hippocampal and cortical neurons, where PDE2A contributes efficiently to the degradation of glutamate-induced cGMP signal (Giesen et al., 2020). However, and in agreement with its low affinity for cAMP, PDE2A only regulates high cAMP levels such as those reached during peak cAMP response to D1 receptor stimulation, or elevated steady-state cAMP levels induced by the activation of adenosine A_{2A} receptors (Mota et al., 2021).

The fragile X syndrome (FXS) is a neurodevelopmental disease leading to inherited intellectual disability. The FMR1 gene encoding the fragile X mental retardation protein (FMRP) is not expressed in this disorder. FXS patient and Fmr1-KO mice have a deficit in the regulation of mRNA translation in neuronal cells. One target of FMRP is PDE2A (Maurin et al., 2018) and in Fmr1-KO mice, enhanced PDE2A activity was observed in hippocampal slices expressing the cAMP biosensor Epac-S^{H150} (Maurin et al., 2019). Pharmacological inhibition of PDE2A durably reversed the behavioral phenotype of Fmr1-KO mice, suggesting a novel therapeutic strategy for the treatment of mental retardation.

In peripheral neurons, PDE2A also plays an important role in the regulation of cardiac neurotransmission (Liu et al., 2018; Li and Paterson, 2019). More precisely, in cardiac stellate ganglia, brain natriuretic peptide (BNP) efficiently regulates norephinephrin release, through an increase in cGMP signaling. In rats and human patients with sympathetic hyperactivity, the effect of BNP/cGMP signaling is reduced due to enhanced PDE2A activity. Interestingly, inhibition of PDE2A activity restored norepinephin levels induced by the BNP/cGMP, suggesting that PDE2A may be a therapeutic target to reduce sympathetic hyperactivity (Liu et al., 2018).

PDE1B: mediator of Glutamate/Ca2+ signaling

PDE1, which is activated by calcium-calmodulin, is highly expressed throughout the brain. PDE1 degrades both cAMP and cGMP, with a relatively low substrate affinity. While cGMP was produced as a result of calcium influx and nNOS activation, this intracellular calcium also activated PDE1 which largely contributed to the degradation of cGMP, in a negative feedback loop (Giesen et al., 2020). The details of PDE1 action was also studied in brain slice preparations where NMDA uncaging transiently decreased cGMP or cAMP levels, an effect lost in the presence of a highly selective PDE1 inhibitor (Betolngar et al., 2019). Such transient PDE1 activation may be of particular importance when dopamine is released simultaneously with glutamate or with a post-synaptic action potential, a situation known to induce synaptic plasticity. Indeed, in striatum slices, simultaneous release of dopamine and NMDA showed that PDE1 activity reduces the amplitude of the D1-mediated cAMP response. Finally, synaptic plasticity appeared enhanced when PDE1 was inhibited, suggesting that PDE1 inhibition may be a novel therapeutic strategy to treat cognitive deficits (Li et al., 2016; Snyder et al., 2016).

Spatial and temporal integration

cAMP/PKA compartmentation in developing neurons

Even though cAMP is a small water-soluble molecule that diffuses freely in the cytosol, the propagation of a cAMP signal throughout the cell is severely constrained by the highly efficacious degradation mediated by phosphodiesterases. Since cAMP is produced at the plasma membrane and degraded by membrane and cytosolic PDEs, local cell geometry - i.e. surface to volume ratio - plays a critical role in the propagation of this signal. This is of particular importance in the case of neurons which bear cellular compartments of widely different sizes. In addition, local changes in the density of signaling enzymes adds a further level of complexity to signaling compartmentation. For example, PDE interaction with scaffolding proteins, such as AKAP, favors the local degradation of cAMP and efficiently control the spread of cAMP around its production site. This regulation was observed in a recent study in which an AKAR sensor was targeted to the peri-nuclear compartment in embryonic cultured neurons. This work revealed an mAKAP-PKA-PDE4D complex at the peri-nuclear membrane that efficiently controls neurite growth and neuron survival (Boczek et al., 2019).

Spatially regulated cAMP signals were also observed in the axonal compartment, in early stages of the embryonic development. At later developmental stages, this axonal compartmentation achieved by AKAP-PDE is reduced, and the cAMP signal rises locally in the axon favoring axonal growth (Averaimo et al., 2016; Gorshkov et al., 2017).

Another important sub-cellular compartment in immature migrating neurons is the primary cilium. This small organelle forms close to the centrosome and changes dynamically during the various migration steps. The cAMP biosensor Epac-S^{H187} revealed that this primary cilium is associated with a cAMP hot spot that forms transiently in the leading process of the migrating neuron, in synch with the different steps of cell movement (Stoufflet et al., 2020).

Membrane - cytosol - nucleus attenuation in most neurons

In pyramidal cortical neurons or intralaminar thalamic neurons, cAMP levels in the somatic cytosol remains relatively low, in the submicromolar range, in response to the activation of a wide range of GPCR, such as serotonin 5HT7, dopamine D1, β1-adrenergic or neuropeptide receptors (Gervasi et al., 2007; Castro et al., 2010; Hu et al., 2011; Castro et al., 2013; Nomura et al., 2014). This low cAMP response is sufficient to trigger PKA activation, that differed in amplitude and kinetics depending on the compartment where the signal was recorded (Figure 1A). For example, in thin dendrites where the biosensor reports the signal mainly in the sub-membrane compartment, cAMP accumulates easily and activates PKA, but its concentration fades away in the somatic cytosol and large primary dendrites (Castro et al., 2010; Luczak et al., 2017; Tang and Yasuda, 2017). Electrophysiological recordings of a PKA-sensitive potassium channel confirmed that full and fast PKA activation occurs at the sub-membrane domain. This effect has been theoretically predicted and would be favored by a higher surface to volume ratio, leading to cAMP accumulation in a confined space and its functional coupling with PKA and target channels (Neves et al., 2008; Luczak et al., 2017). It must be noted that when the AKAR biosensor is targeted to microtubules, the time-course of the biosensor is faster - similar to the time-course of sAHP modulation - than the AKAR signal measured in the bulk cytosol, illustrating the importance of sub-cellular compartmentation of PKA action (Ma et al., 2018).

In pyramidal cortical neurons and thalamic neurons, the targeting of the AKAR sensor to the nuclear compartment clearly shows that the PKA signal in the nucleus is further dampened in both amplitude and kinetics, with more than 10 minutes being required to get a sizable nuclear response (Gervasi et al., 2007) (Figure 1B). This effect is in agreement with the passive diffusion of the catalytic subunit of PKA through the nuclear pore (Hagiwara et al., 1993; Harootunian et al., 1993) as well as high phosphatase activity. Indeed, type 1 phosphatase (PP-1) efficiently counteracts PKA activity in the nuclear compartment, and is probably responsible for the lower sensitivity of these neurons to initiate gene expression in response to brief dopamine stimulation (Castro et al., 2013; Yapo et al., 2018).

In the cytoplasm and sub-membrane compartment, the major negative control of intracellular cAMP is achieved by PDE4. In thin dendrites, PDE4 tightly controls tonic cAMP production, PKA activation and consequently neuronal excitability (Castro et al., 2010, 2013). This regulation of tonic cAMP level probably allows for multiple possibilities of signal integration and cross-talks with other cascades. Since PDE4 inhibitors can render the neurons responsive to low levels of agonist, it probably restores or amplifies the postsynaptic response when neuromodulator levels fall or lose part of their effect, such as in depressive or memory disorders (Bolger, 2017; Blokland et al., 2019).

Overall, this profile of rapid and powerful cAMP/PKA responses at the sub-membrane compartment that progressively fades as the signal progresses through the cytosol and into the

nucleus is the most commonly accepted mechanism for cAMP/PKA signaling and was also reported in many other cell types (DiPilato et al., 2004; Sample et al., 2012; Haj Slimane et al., 2014; Yang et al., 2014).

Specific dynamic features of striatal neurons

In vivo, dopamine release in the striatum lasts less than a second, but is sufficient to modulate striatal plasticity and trigger the induction of gene expression (Howard et al., 2013). In brain slices, dopamine released from caged precursor or from dopaminergic fibers by optogenetic stimulation induces a robust PKA response (Yapo et al., 2017; Ma et al., 2018). This transient release of dopamine triggers transient and opposite cAMP/PKA responses in D1 and D2 MSNs over a time scale of a few minutes. Surprisingly, the profile of signal transduction in MSNs profoundly differ from what was observed in cortical pyramidal neurons, described above (Figure 1A-C). D1-type MSNs exhibit much faster, larger, and longer lasting cytosolic cAMP and PKA responses (Castro et al., 2013). This high sensitivity is most likely related to the expression of an unusual set of signaling enzymes in MSNs, such as Gαolf instead of Gs, type 5 adenylyl cyclases (Matsuoka et al., 1997), PDE10A (Seeger et al., 2003; Coskran et al., 2006; Lakics et al., 2010) and dopamine-and cAMP-regulated phosphoprotein 32 KDa (DARPP-32)(Nishi et al., 2011). DARPP-32, when phosphorylated by PKA, becomes a strong inhibitor of type 1-phosphatase (PP-1) activity (Hemmings et al., 1984; Svenningsson et al., 2005). This positive feed-forward control amplifies PKA response by increasing the duration of cytosolic PKA responses to transient dopamine in D1 MSNs (Castro et al., 2013). DARPP-32 is also required for spine enlargement (Yagishita et al., 2014). Such mechanism also promotes a peculiar all-or-none responsiveness in the nuclear compartment and was associated with the expression of c-fos (Yapo et al., 2018) (Figure 1D). Biosensor imaging and computer modeling suggest that striatal PKA follows the classic mechanism of passive diffusion of the catalytic sub-unit from the cytoplasm through the nucleus, but with an additional amplification of its own diffusion in the nuclear compartment at the level of the nuclear pore. An additional amplification mechanism is based on PP-1 inhibition in the nucleus, possibly mediated by DARPP-32. Such positive feed-forward control loop clearly contributes to produce all or none responses to dopamine (Yapo et al., 2018).

Beyond these specific biochemical features, neuronal geometry also adds more complexity to the integration of dopamine signals. An observation of importance is that, depending on the location where the cAMP/PKA signal is initiated in the dendritic tree, its propagation through the dendritic tree towards the nucleus does not follow the expected linear diffusion pattern (Li et al., 2015). In another study, the authors analyzed dendritic spine enlargement following the coincident release of glutamate, dopamine, and post-synaptic action potential (Yagishita et al., 2014). This effect requires DARPP-32, as well as the calcium-activated adenylyl cyclase 1. This work also reports a decrease in the PKA response to dopamine release as the distance from the cell body increases, an effect that depends on PDE10A activity. This is the opposite of what was observed in the cortex, possibly because PDE10A is associated with the membrane whereas PDE4 is cytosolic. Most importantly, spine enlargement was only observed when preceded in less than 1 s by a calcium signal, and biosensor imaging revealed the successive steps for achieving this temporal specificity. This highlights the importance of the

precise timing and spatial localization of signaling events in a fundamental process involved in learning and memory.

Perspectives

There are many steps in the integration of an extracellular neuromodulatory signal to a change in phosphorylation of a target protein, and each of these steps allow for cross-talks with other signaling pathways. These events take place in different sub-cellular compartments, with different geometries and different equipments in signaling enzymes. In most cases, the signal gets progressively attenuated during its journey through different cell compartments, consistent with homeostasis tending to reduce any deviation from equilibrium. MSNs clearly depart from this logic by integrating very efficiently brief dopamine signals - a core feature of reward-mediated learning - into powerful cAMP/PKA signals, a feature associated with the expression of unusual signaling enzymes. Further work is needed to explore other brain regions and determine if other specific neurobiological functions are associated with other peculiarities of integration mechanisms.

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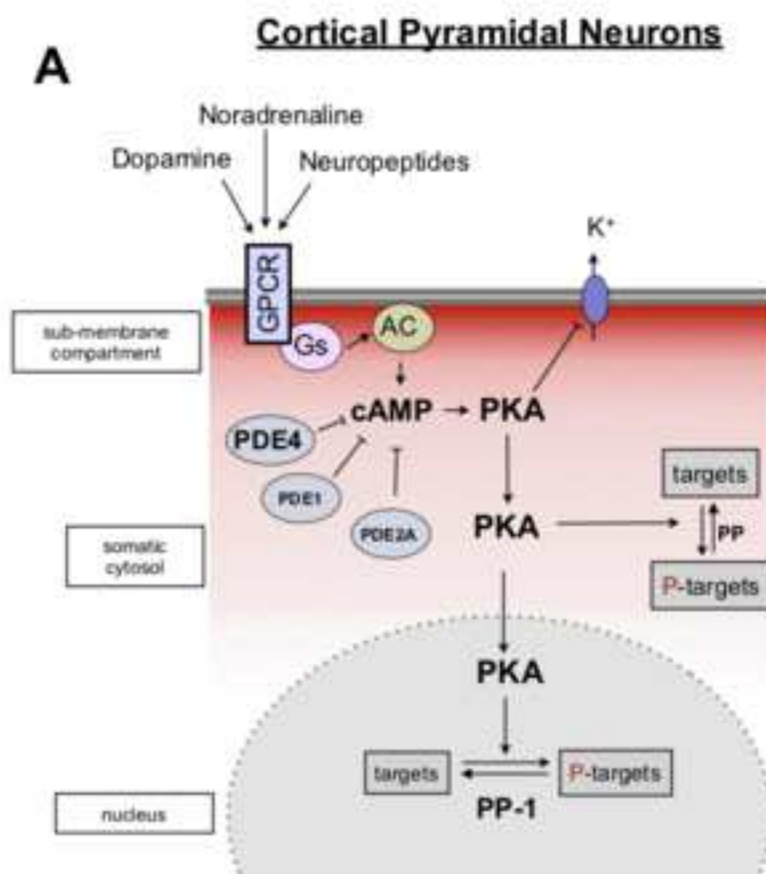

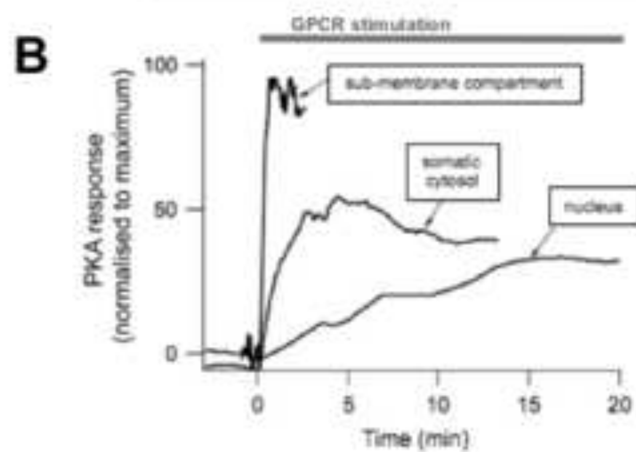

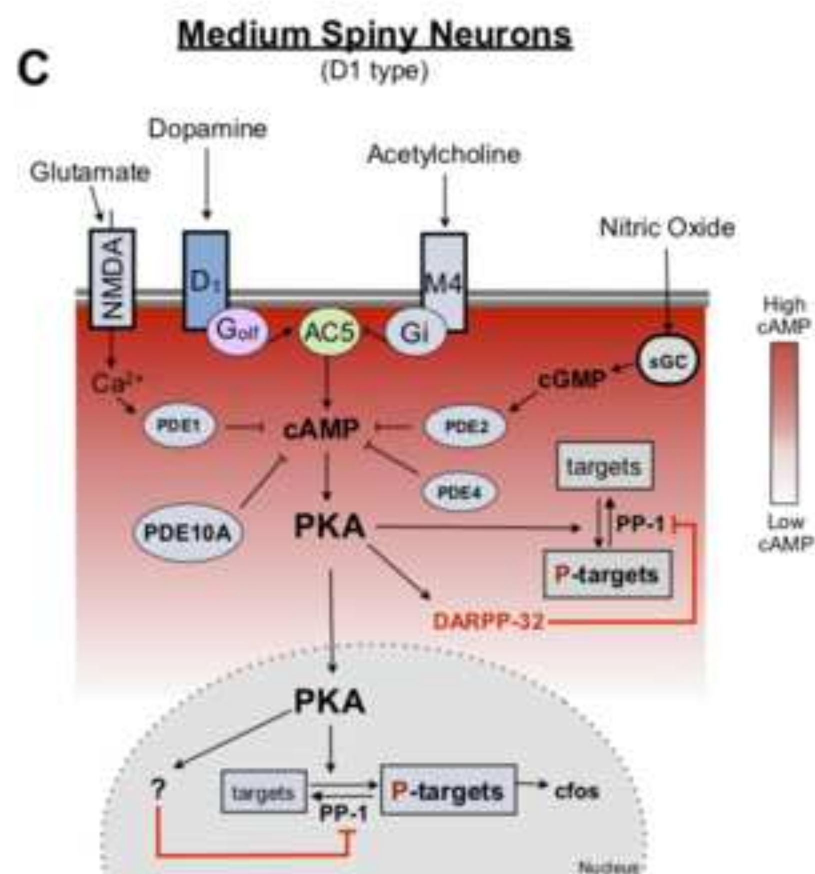

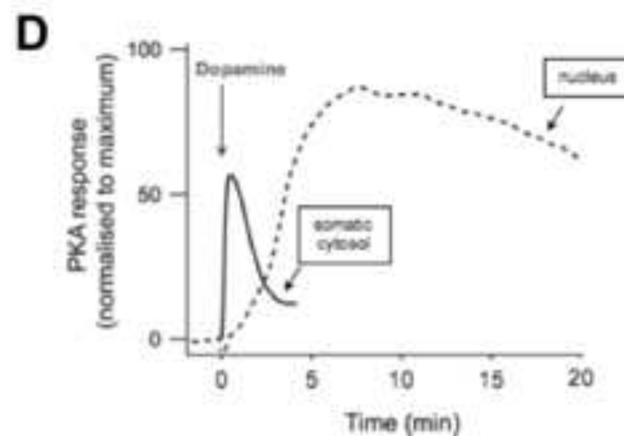

Figure 1

Figure 1: Schematic representation of the cAMP/PKA signaling pathway and its regulation in pyramidal neurons of the cortex and in medium-sized spiny neurons (MSNs) of the striatum. A) In cortical pyramidal neurons (as in many other cell types), G protein coupled receptor stimulation results in a moderate increase in cAMP level and PKA activation, that is progressively attenuated as it diffuses from the membrane through the cytosol and into the nucleus. B) PKA-dependent phosphorylation is measured electrophysiologically in the sub-membrane compartment through the inhibition of a potassium current, using the AKAR2 biosensor in the somatic cytosol and using AKAR2-NLS in the nucleus, showing decreasing efficacy and slower action. Copyright 2007 Society for Neuroscience. C) Striatal D1-type medium spiny neurons (MSNs) can detect brief dopamine release and induce a large increase in cAMP/PKA signaling with a specific temporal profile. D) Dopamine (1 µM) was released from a "caged" precursor by a flash of UV light while PKA-dependent phosphorylation was monitored in the cytosol with AKAR3 (plain trace) and in the nucleus with AKAR2-NLS, as described in (Yapo et al., 2018): in contrast with the cortex, a brief cytosolic signal of moderate amplitude is associated with a much larger and longer lasting nuclear signal.