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Update article

Optogenetic neuromodulation: New tools for monitoring and breaking neural circuits☆

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ABSTRACT

Optogenetics is the combination of optical tools to monitor (i.e. "reporters") or interfere (i.e. "actuators") with neural activity, and genetic techniques to restrain the expression of these reporters and actuators in the neuronal populations of interest. Such combination of optical and genetic tools, together with the emergence of new animal models such as the zebrafish larva, has proven extremely valuable is dissecting neural circuits. Optogenetics provide a new framework to address issues that are fundamentally dynamic processes, such as sensorimotor integration in the vertebrate spinal cord. By shifting from spatially targeted electrical stimulation to genetically targeted optical stimulation, optogenetic also opens new avenues for innovative neurorehabilitative strategies, in particular after spinal cord injury.

1. Introduction

A critical step in order to understand neural circuits is the ability to selectively monitor and interact with populations of neurons. Until recently, electrophysiological techniques allowed recording from and stimulating only a limited number of morphologically identified neurons from ex-vivo tissue samples or paralyzed animals. Optogenetics, which is the combination of optical tools and genetic targeting techniques, has revolutionized this paradigm by allowing both monitoring and stimulation or inhibition of genetically identified populations of neurons, in paralyzed but also in moving animals.

Optogenetic neuromodulation relies on optical "reporters", which emit light when the cell is active, to inform us on the activity of the population of neurons that has been genetically targeted. On the other hand, optical "actuators", which have the ability to depolarize or hyperpolarize the neurons in which they are expressed, allow us to manipulate neural circuits with light. The combination of optical reporters and actuators with advanced genetic techniques to target their expression in precise populations of neurons provides new avenues to break neural circuits and ultimately understand their function in intact vertebrates.

2. Reporters: monitoring neural circuits

Monitoring neural activity can be indirectly achieved by measuring the intracellular level of calcium, since electrical activity of neurons lead to a calcium influx through voltage dependent calcium channels [1]. This strategy has led to the elaboration of number of chemical calcium indicators and genetically encoded calcium indicators (GECI) that have been successfully used in many different mammalian and non-mammalian animal models [2,3] (Fig. 1A1). GECIs consist in engineered fluorescent proteins having two key features: their emission properties are modified depending upon the intracellular level of calcium, and their pattern of expression can be restricted using the above mentioned genetic toolbox. They include either permutated single fluorescent proteins whose fluorescence properties are modified when calcium is binding to Ca^{2+} recognition elements [4], or pairs of fluorescent proteins in which conformational change induced by calcium binding leads to Förster Resonance Energy Transfer (FRET) mediated modification of fluorescence [5].

The transparency of the zebrafish larva and its genetic accessibility make it an ideal model to use such optical tools for monitoring neural activity. In the first zebrafish study using a GECI (cameleon), expressed under the islet-1 promoter [6] (see section 3.1.2), calcium transients could be observed within the spinal cord, in Rohon-Beard neurons activated by electrical cutaneous stimulation, and in motoneurons and Commissural Descending (CiD) interneurons during escapes triggered by a mechanical head tap [7]. Since this first study, GECIs have been extensively used in

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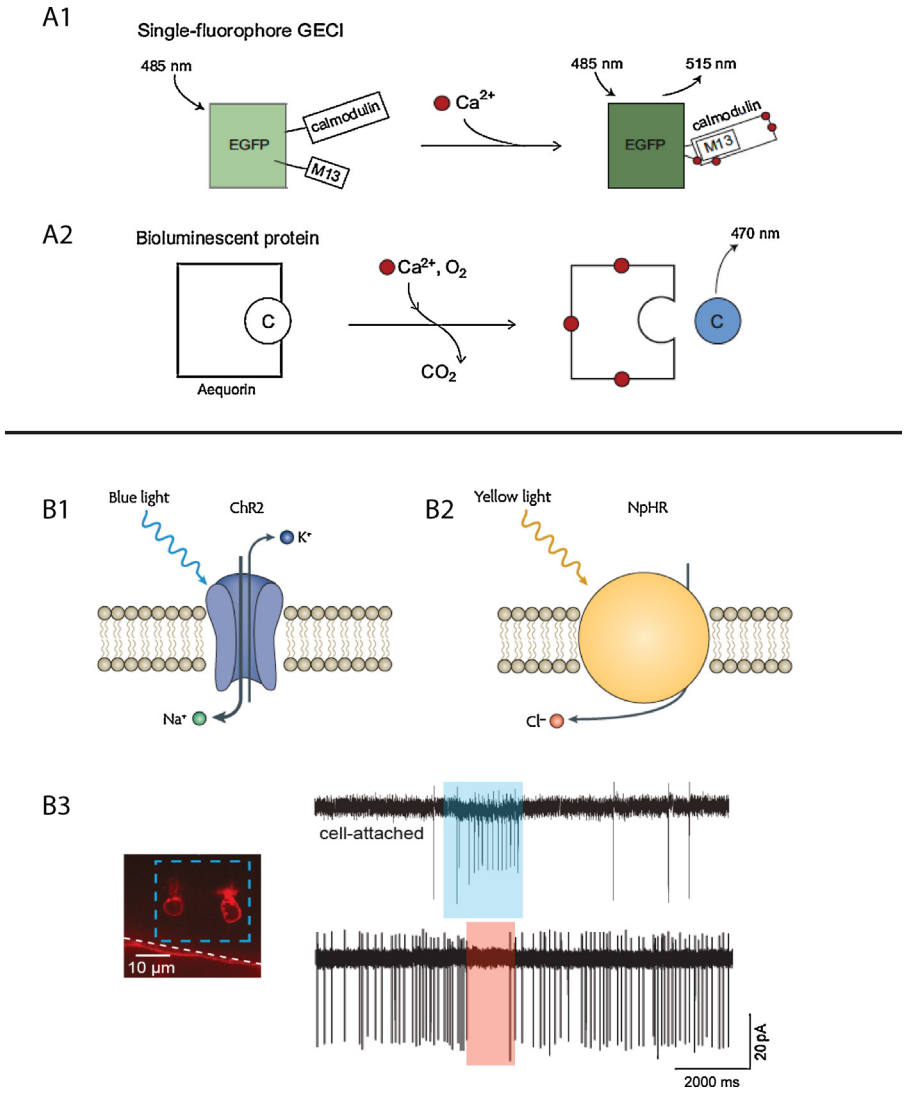

Fig. 1. Monitoring and breaking neural circuits with genetically encoded reporters and actuators. A. Calcium indicators. A1. Genetically encoded calcium indicators (GECIs) allows for monitoring neural activity through changes in intracellular calcium concentration. In a single-fluorophore GECI, such as GCaMP, conformational modification upon calcium binding is intra-molecular, leading to an increase in the emitted fluorescence (515 nm). A2. Bioluminescent GECIs, such as aequorin, binding of calcium ions leads to oxidation of coelenterazine. Chemiluminescence resonance energy transfer (CRET) between aequorin and GFP is responsible for the emission of a green photon. Adapted from Grienberger et al., 2012. B. Optogenetic actuators. Following illumination with blue light (470 nm, blue pulses), channelrhodopsin-2 allows the entry of cations into the cell (B1), triggering action potentials in whole-cell current-clamp. Following illumination with yellow light (580 nm, yellow line in B3), halorhodopsin pumps chloride anions (B2), leading to neural silencing (B3). Adapted from Zhang et al., 2007.
B3: figure courtesy of C. Stokes.

zebrafish to monitor neural activity in various behavioral paradigms, including investigating the role of the optic tectum in prey capture [8], performing brain-wide monitoring of neural dynamics in a sensorimotor virtual environment [9] or testing neural coding of odors by the olfactory bulb [10]. Targeted mutagenesis and high-throughput screening have led to the continuously improvement of GECIs such as the single-fluorophore GCaMP family by optimizing their calcium affinity, kinetics and dynamic range [2,3,11,12]. From the first GCaMP [3] to the current GCaMP6 [13], and including the generation of multi-color variants [14], the always improving GECIs arsenal now allow for monitoring of neural activity over a wide range of firing rates.

One major limitation of GECIs such as GCaMP, regarding in particular investigation of closed-loop sensorimotor behaviors in vivo, is the need for providing focal excitation to the fluorescent proteins. Indeed, this limitation implies constraining the neurons of interest to a given focal plane, either by partially embedding and/or paralyzing the animal. One alternative approach is to use the bioluminescent protein-aequorin-GFP, derived from the jellyfish Aequorea victoria [15] (Fig. 1A2). ApoAequorin, the naturally occurring complex of aequorin with GFP, binds to its substrate coelenterazine, which is then oxidized in the presence of calcium leading to the emission of a green photon by the GFP through chemiluminescence resonance energy transfer (CRET) [16]. Bioluminescence assays based on aequorin-GFP have been used for noninvasive monitoring of neural activity in vitro [17], but also in restrained flies [18] and freely behaving mice [19].

Taking advantage of this bioluminescence approach, monitoring of neural activity in freely behaving zebrafish larvae has been achieved by genetically targeting the expression of aequorin-GFP in a specific subset of neurons and simultaneously counting the number of photons emitted over time while recording the locomotor activity using a high-frequency camera [20]. Remarkably, the author could monitor the activity of a small group of hypocretin-positive neurons in the hypothalamus over several days, or combine a gated photomultiplier tube with stroboscopic

illumination to record visually evoked behaviors [20]. While the aequorin allows for noninvasive monitoring of an entire population of neurons in a moving animal, it does provide any spatial information, thus making the specificity of the genetic targeting a crucial limitation.

3. Actuators: breaking neural circuits

Besides monitoring neural activity, the optical and genetic accessibility of the zebrafish larva also constitute an optimal playground for optogenetic actuators, making it possible to selectively activate or inhibit genetically targeted neurons [21–23]. Channelrhodopsin-2 (ChR2) is a light-gated channel derived from the unicellular alga Chlamydomonas reinhardtii allowing non-specific influx of cations when illuminated with blue light [24,25] (Fig. 1B1). ChR2 can therefore be used to control a genetically targeted neuronal population with a millisecond-timescale precision in a dynamic and reversible manner [26]. First tested in zebrafish to trigger escape responses by photo-activating Rohon-Beard neurons [27], ChR2 has subsequently been used to investigate diverse behaviors such as the optokinetic response [28] or odor responses modulation [29]. Synthetic excitatory actuators, obtained by combining a chemical ligand to a ionic channel, such as the light-gated ionotropic glutamate receptor (LiGluR, [30,31]) and the light-gated metabotropic glutamate receptor (LimGluR2, [32]) have been successfully used to trigger neural activity in zebrafish. For instance, the potential role of Kolmer-Agduhr interneurons in modulating slow locomotion could be investigated by combining LiGluR activation and Gal4/UAS enhancer-trap transgenics [33].

Optogenetics have also been used to selectively silence genetically targeted neurons in zebrafish, using the light-gated chloride pump halorhodopsin (NpHR), derived from the archaebacterium Natronomonas pharaonis [34,35] (Fig. 1B2). NpHR hyperpolarizes neurons by pumping chloride ions upon activation with yellow light, leading to optical silencing. Interestingly, optical silencing with NpHR, and its improved variant eNpHR [36], can be combined with photo-activation using ChR2 to provide a versatile optogenetic toolbox to dissect circuits within the same animal [37].

Such combined strategy has been successfully used in zebrafish to identify neurons in the hindbrain able to initiate locomotion through a rebound activity after eNpHR silencing [38], or dissecting the mechanism of eye saccades during optokinetic response [28]. In those two studies, light was delivered using optic fibers to achieve a high spatial selectivity regarding the stimulated area. However, new microscopic techniques relying on light patterning with multi-mirror devices [10,39] or temporal focusing of two-photon excitation [40] should allow for more complex 2D stimulation patterns. Lastly, 3D optical stimulation with a high spatiotemporal resolution could be achieved by combining digital holography and temporal focusing [41], opening the way for simultaneous imaging and neural manipulation in multiple planes in vivo [22].

4. The escape response of the zebrafish as a model for sensorimotor integration

The "escape response" is a stereotyped sensorimotor behavior whereby the animal aims to escape an approaching predator, which has been extensively described in many teleost fish species, including the goldfish and zebrafish [42], but also in other anamniotic vertebrates such as the lamprey [43] or the Xenopus tadpole [44]. Escape responses in zebrafish can be elicited by several types of sensory stimuli, such as touch to the head or the tail [45], a water jet to the otic vesicle [46] or an auditory-vestibular stimulus produced by a sound vibration for instance [47]. In the zebrafish larvae aged 6 to 9 days post-fertilization (dpf), it typically consists in an initial fast "C-shaped" bend, followed by a counter-bend in the opposite direction, and lastly a burst swim [48] (Fig. 2A). Typical kinematics parameters for escapes in zebrafish larvae are: a mean angular velocity of 21.2°/ms, a mean duration until completion of the first bend of 10.4 ms, a mean counter-bend angle of 125.1° [48].

5. Monitoring spinal neurons during active locomotion

The ability to simultaneously record active locomotor behavior and monitor neural activity in partially restrained zebrafish has proven very valuable to dissect the descending motor and sensory control of escape responses. Similar head-embedded experimental paradigms have also been used to investigate the recruitment of spinal interneurons during active locomotion [45,49]. Although studies based on calcium imaging of either hindbrain or spinal neurons in partially restrained animals has been an important step forward in the study of sensorimotor behaviors such as the escape response, they did not provide information about neural activity in the moving tail of the fish, therefore discarding segmental sensory feedback due to locomotion itself.

However, new techniques such as bioluminescent monitoring of genetically targeted neurons with aequorin-GFP could prove helpful in providing specific monitoring of neural activity in actively moving animals, whether head-restrained or freely swimming. Indeed, using an experimental setup adapted from Naumann et al. in which escape responses were elicited in head-embedded zebrafish larvae either by a water jet to the otic vesicle or an auditory-vestibular sound stimulus, we can simultaneously record detailed quantitative kinematics parameters and count photons emitted by the aequorin-GFP. Taking advantage of the Gal4/UAS system to restrict the expression of aequorin-GFP to motoneurons, we could obtain bioluminescence signals following the recruitment of spinal motoneurons (Fig. 2D, Knafo et al. unpublished). This approach could prove particularly useful to investigate the recruitment of sensory spinal neurons during active locomotion, and question whether sensory feedback from the moving part of the tail does actually modulate locomotion.

6. Implications for neuromodulation strategies after spinal cord injury

The emerging concept that intrinsic spinal circuits can produce adaptive locomotion through modulation by sensory feedback, and do so independently, at least to some extent, from supra-spinal inputs, bears important consequences for new neurorehabilitative strategies after spinal cord injury.

Results have been obtained recently in rodents [50], in which recovery of coordinated hindlimbs locomotion on a treadmill could be achieved only 1-week after complete thoracic (T7) spinal transection when combined lumbosacral electrical epidural stimulation (EES) and systemic application of serotoninergic agonists were applied [51]. Interestingly, removing peripheral sensory inputs by unilateral dorsal rhizotomy prevented EES-facilitated locomotor recovery after complete spinal transection, but only on the deafferented side, thereby confirming the hypothesis that sensory feedback drives the reorganization of intrinsic spinal circuitry [52].

However, those results only concerned treadmill-induced "automatic" locomotion. To what extent can we exploit the plasticity of spinal sensorimotor circuits to induce restoration of voluntary locomotion? This question was investigated by a recent study [53], in which the authors used a simultaneous dual hemisection paradigm in adult rats together with a so-called

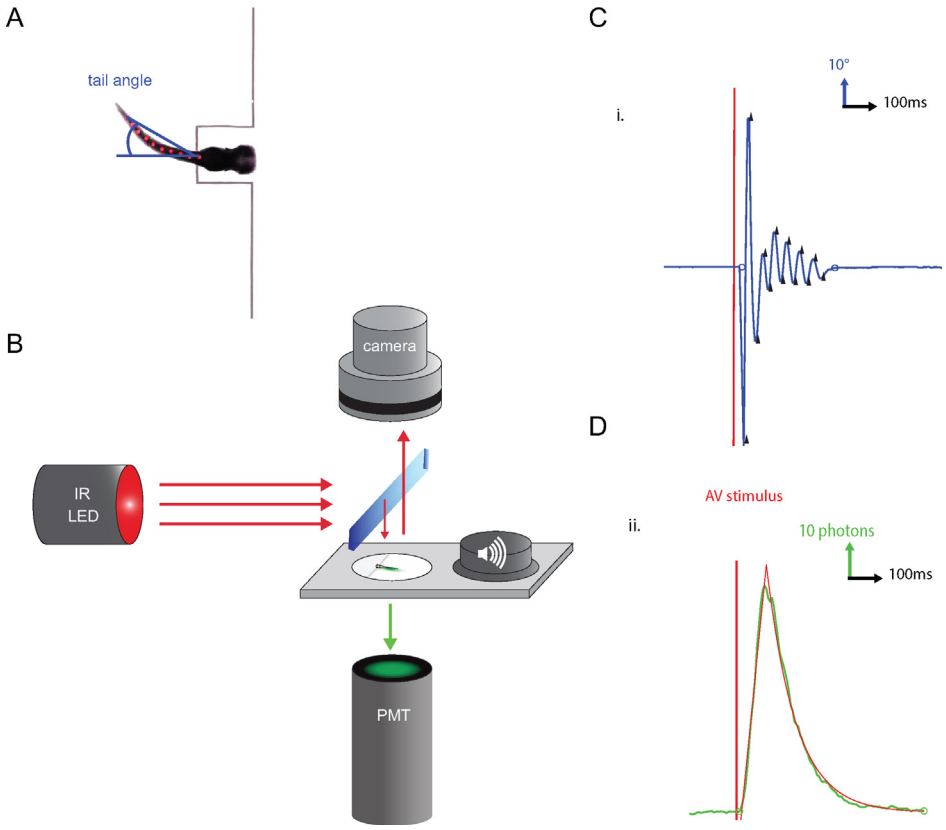

Fig. 2. Monitoring the activity of spinal neurons during active escape responses in zebrafish. A. A typical escape in a zebrafish larva, with the tail angle over time tracked using a custom video analysis program. B. A setup for simultaneously recording active locomotion using a high-speed camera, while counting photons emitted by spinal motoneurons during escape responses a the transgenic line selectively targeting spinal motoneurons. C. In blue: tail angle (in degree) between the first and last points of the tail over time. D. In green, the bioluminescent signal over time (number of photons emitted/10 ms), reflecting the neural activity of spinal motoneurons during free-tail escapes in the zebrafish larva.

"electrochemical neuroprosthesis" (i.e. the combination of lumbosacral epidural electrical stimulation together with systematic administration of a cocktail of monoaminergic agonists). They observed that rats trained with a robotic postural interface encouraging supra-spinally mediated locomotion could regain voluntary control through remodeling of corticospinal projections. A similar approach has even been used successfully in a paraplegic human subject, who could regain some voluntary control of one of his lower extremities after intensive rehabilitation and electrical epidural stimulation, although this recovery was very limited and observed in few individuals [54,55].

These results have raised hopes that clinically significant locomotor recovery can be achieved through reorganization of intrinsic sensorimotor circuitry, facilitated by intensive training and electrical and/or chemical manipulation. However, one major issue of such studies is that they can probe changes in spinal circuitry only in a very indirect manner. Optogenetic tools, by providing a means to dissect sensorimotor circuits in the spinal cord in a dynamic fashion, could solve such limitation. Besides, genetically targeted optical rather than spatially targeted electrical stimulation represents a truly paradigm shift in the field of neuromodulation.

7. Conclusion

The ability to monitor active behaviors in vivo with precise kinematics also provides a new framework in which results obtained from fictive recordings could be validated in order to confirm their environmental relevance. Moreover, the variability observed in real-world locomotor behaviors also questions whether "hard-wired" connectivity diagrams are actually the most suitable mean of modeling sensorimotor integration [56]. The emergence of multifunctional neuronal populations, i.e. neurons that are recruited during multiple behaviors [57], as opposed to specialized neurons that are only active for a given motor output [47], will also benefit from in vivo studies involving active locomotion, in which multiple behaviors can be tested within the same animal [58].

The advances in genetic targeting and the identification of molecular markers to classify homologous populations of spinal neurons have allowed bringing together results obtained across animal models. However, the extent to which the walking CPG of mammalian vertebrates (such as rodents and cats) and the swimming CPG of non-mammalian vertebrates (such lampreys, zebrafish or tadpoles) can mutually inform each other remains unclear. In this regard, amphibian metamorphosis, during which the swimming CPG of a tadpole is transformed into a frog walking CPG, could provide an intriguing and unique model [59].

Sensorimotor behaviors are inherently a closed-loop process, where sensory feedback heavily influence the motor output. Although spinal networks do integrate this sensory information to modulate locomotion, detailed access to spinal sensorimotor circuitry has so far been only possible in open-loop preparations, where the sensory feedback was not taken into account. New tools, such as optogenetic reporters and actuators, combined to genetically accessible animal models, such as zebrafish, should provide bright opportunities for monitoring targeted spinal

sensorimotor neurons in actively moving animals, and, possibly, closing the loop.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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