1.3.20 *In vivo* brain physiology: molecules and brain disease

The clinical manifestations of the pathological brain include epilepsy, cognitive deficits and impairment of superior functions. We are gradually realizing that this variety of signs arises from defects at the molecular level that impair cortical-circuit formation and function. We study the biophysical basis of brain disease by applying a variety of techniques for the i- vivo study of cortical processing. To this end we are also developing several tools useful for the study of intracellular signaling and for the creation of mouse models of brain diseases.

Biophysics of the neuronal negative feedback by means of in vivo two photon non-linear microscopy and spectroscopy

Proper brain computation requires a fine equilibrium between excitatory connections, necessary to recruit large neuronal ensembles responsible for computation and behavioral responses, and the negative feedback that maintains activity within the rather limited dynamic range available to neurons. The inhibitory feedback is provided by the activity of a specific set of inhibitory synapses permeable to chloride. The direction of chloride flux determines the extent of neuronal inhibition and these currents depend critically on the intracellular concentration of chloride. In a set of studies, we have achieved methodological and basic-science advancements in this important field.

The excitation/inhibition equilibrium is a dynamic process in continuous evolution during daily activity and during sleep. The key effector of inhibition is a class of inhibitory neurons that uses gamma amino butyric acid (GABA) as a neurotransmitter. When GABA is released at the synapse a chloride channel opens and, if intracellular chloride is low enough, the entrance of negative charges renders the neuron more negative thus opposing the effects of excitation (Fig. 1A). A correct operation of this feedback is essential for proper brain computation as demonstrated by the fact that disruptions of this system leads unavoidably to some form of brain pathology. Given that inhibitory currents are mostly carried by chloride, its intracellular concentration ($[Cl]_i$) dictates the direction of these currents and the result of their action: low $[Cl]_i$ leading to inhibition and high $[Cl]_i$ leading to depolarization. The regulation of $[Cl]_i$ exerts wide-ranging effects on synaptic signaling and plasticity and on development and disorders of the brain. In the last few years, we developed a technique for the measurement of $[Cl\text{-}]_i$ by means of two photon imaging and spectroscopy. Our genetically encoded fluorescent sensor includes a spectroscopic reference (Fig. 1) and we were able to perform parallel measurements of $[Cl]_i$ and pH at the single-cell level in the mouse cortex in vivo.

With this too we have demonstrated for the first time the developmental change of intracellular chloride in the cortex [8] and how this process is altered in a mouse model of cognitive impairment.

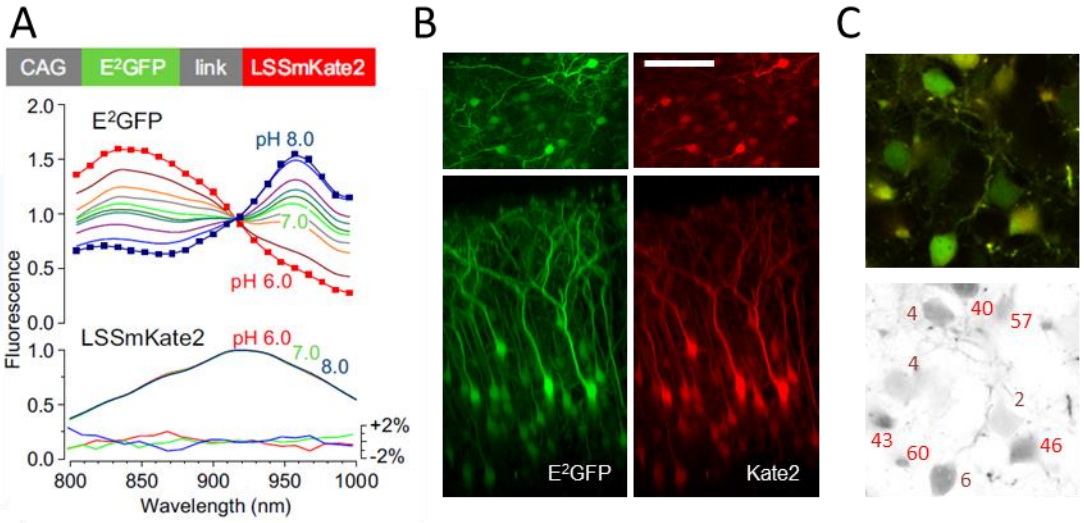

Figure 1. A) The chloride/pH sensor is formed by the fusion of the Cl sensitive element E²GFP with the insensitive spectral reference LSSmKate2. E²GFP is sensitive to both intracellular pH and $[Cl]_i$. Changes in pH are reported with a shift of the 2-photon excitation spectra. Chloride interacts with the chromophore by collisional quenching, therefore increasing $[Cl]_i$ is reported as a decrement of the green fluorescence in comparison with the red reference. The plot shows the two-photon excitation spectra of E²GFP and LSSmKate2 at different levels of pH. The non-labeled spectra of E²GFP have been obtained at pH 6.4, 6.8, 6.9, 7.1, 7.2, 7.4, and 7.6. The excitation spectrum of LSSmKate2 is pH-insensitive: the lower panel shows the normalized difference between the spectra obtained at pH values of 6, 7, and 8, with the mean spectrum averaged for all pH values. B) Expression and spectra of LSSmClopHensor in vivo after transfection by in utero electroporation. The field depicted lies in the superficial layers of the visual cortex. Calibration bar 100 µm. C) Measurement of intracellular Chloride in the mouse cortex: different cells display different hues from green to orange, with low chloride cells appearing green. Numbers in the lower panel report the computed concentrations.

ddGCaMP: a new tool for long term imaging of intracellular calcium

Calcium imaging is a powerful tool for the study of intracellular signaling and for the single neuron level analysis of neuronal computation. The main tool of the trade is a family of Ca-sensors formed by proteins that are almost non-fluorescent at resting state and that undergo a large increase in fluorescence upon Ca binding. Therefore, neuronal activity, which causes the influx of calcium ions, is signaled by a transient fluorescence increase. Despite the importance of this tool, its biocompatibility is far from optimal, since the sensor is a Ca binding protein and its prolonged expression leads to an epileptic phenotype, possibly because of altered Ca homeostasis. In this work we have fused GCaMP6 with a destabilization domain that causes the rapid degradation of the protein. This domain can be neutralized by a ligand that crosses the blood brain barrier and rapidly suppresses protein degradation. In this way, we can target the cortex early in development with widespread GCaMP transfection but the protein levels are so low that do not cause any side effect. One hour before of the imaging session, the ligand is provided to the sensor by simple intraperitoneal injection, the degradation stops and the sensor builds up in the cortex (Fig. 2) to return to basal level after a few hours. This tool will allow long term chronic recording of Ca activity during development without any of the side effects due to the constitutive expression of GCaMP. The data shown in Fig 2 are collected with a wide field microscope that we have developed for wide field imaging of the entire brain.

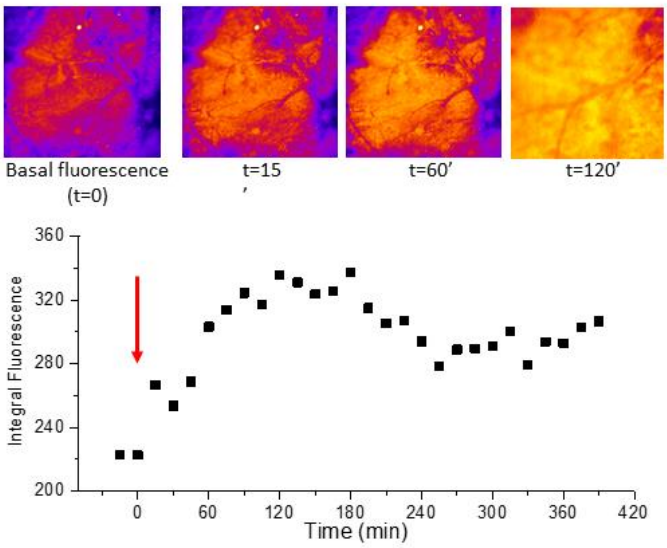

Figure 2. Wide field imaging of the resting state fluorescence of ddGCaMP in basal conditions and after activation (red arrow). The basal florescence is mostly due to background fluorescence and increases rapidly after the blockage of protein degradation (red arrow in the lower plot). The field is about 4 mm wide.

The analysis of wide field imaging data requires specific software tools that can extract the fluorescence fluctuations caused by Calcium transient from the background noise. To this effect, we have developed a novel quantitative method for the concurrent analysis of electrophysiological and 2-photon imaging data. We show how the statistics of the time series provided by the recordings of LFP and Ca^{2+} imaging can be used to classify epileptic activity in the entire zebrafish brain. Then, we demonstrate that by cross correlating the time series originating from Ca^{2+} imaging with the LFP recordings it is possible to pinpoint the specific regional sources of epileptiform activity [13].

Modelling genetic mosaicism

Genetic mosaicism refers to the presence of genetically distinct cellular populations within the same individual. This condition originates from DNA mutations, either monogenic or genome-wide, that may occur through different biological mechanisms (X-chromosome inactivation for X-linked genes or somatic mutations). A pathophysiological role for mosaicism has been initially identified for X-linked disorders (for example Rett syndrome or Xfragile) and then for disorders associated to somatic mutations, leading to focal cortical dysplasia. As our capacity of detecting somatic mosaicism in human patients improved, it gradually emerged that somatic mosaicism plays an important role in brain pathology, where even low frequency of mutation can lead to cognitive disorders and epilepsy. To underline the importance of this mechanism, recent whole exomes studies on genetic samples from families including one ASD offspring have detected the presence of somatic mosaicism in 3-5% of the analyzed samples. The creation of mosaicism requires the use of conditional transgenic mice where the target gene is normally expressed but it can be excised by the presence of the bacterial enzyme CRE recombinase. The transfection of a random set of cells with CRE recombinase therefore creates a mosaic of expression for the specific gene. This approach has a severe drawback due to the lack of control of the mosaicism

degree. Furthermore, the genetic identity of the cells in the transfected area is unknown. To answer these criticalities, at NEST we have generated Beatrix, a new Cre-reporting architecture capable of amplifying and preserving weak or transient Cre events with a tenfold increase in sensitivity compared to canonical reporter structures. Beatrix is a reliable reporter of Cre-mediated recombination and it allows the creation of genetic mosaics of arbitrary degree that are amenable to be imaged in vivo by two-photon microscopy. As a proof of principle of the methodology, we created a mosaic of expression of the autism-related gene PTEN, a constitutive inhibitor of the mTOR pathway, and we describe the anatomical phenotype of this mosaic in the mouse cortex and olfactory bulb (Fig. 3). Finally, we demonstrate that the cortical mosaic is characterized by impaired network activity and by transient episodes of hyperexcitability strongly reminiscent of the electrophysiological signature of the human disease.

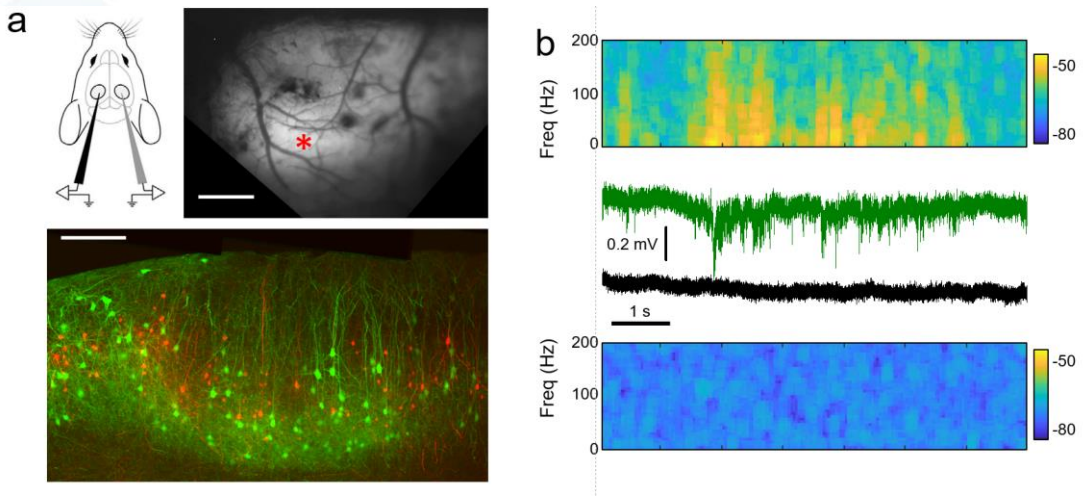

Figure 3. Mosaicism for the gene PTEN leads to an epileptic phenotype. A) double recordings from the focal mutation site and from the opposite, control, hemisphere. The wide field image shows the recording site in correspondence of a fluorescent patch (bar 1 mm). The lower panel shows a coronal section of the brain fixed after the recordings that shows a mosaic of normal neurons (red) and neurons lacking PTEN (green). B) recordings from the focus (green trace) shows characteristics hypersynchronous bursts, while the other hemisphere is silent (black trace).

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