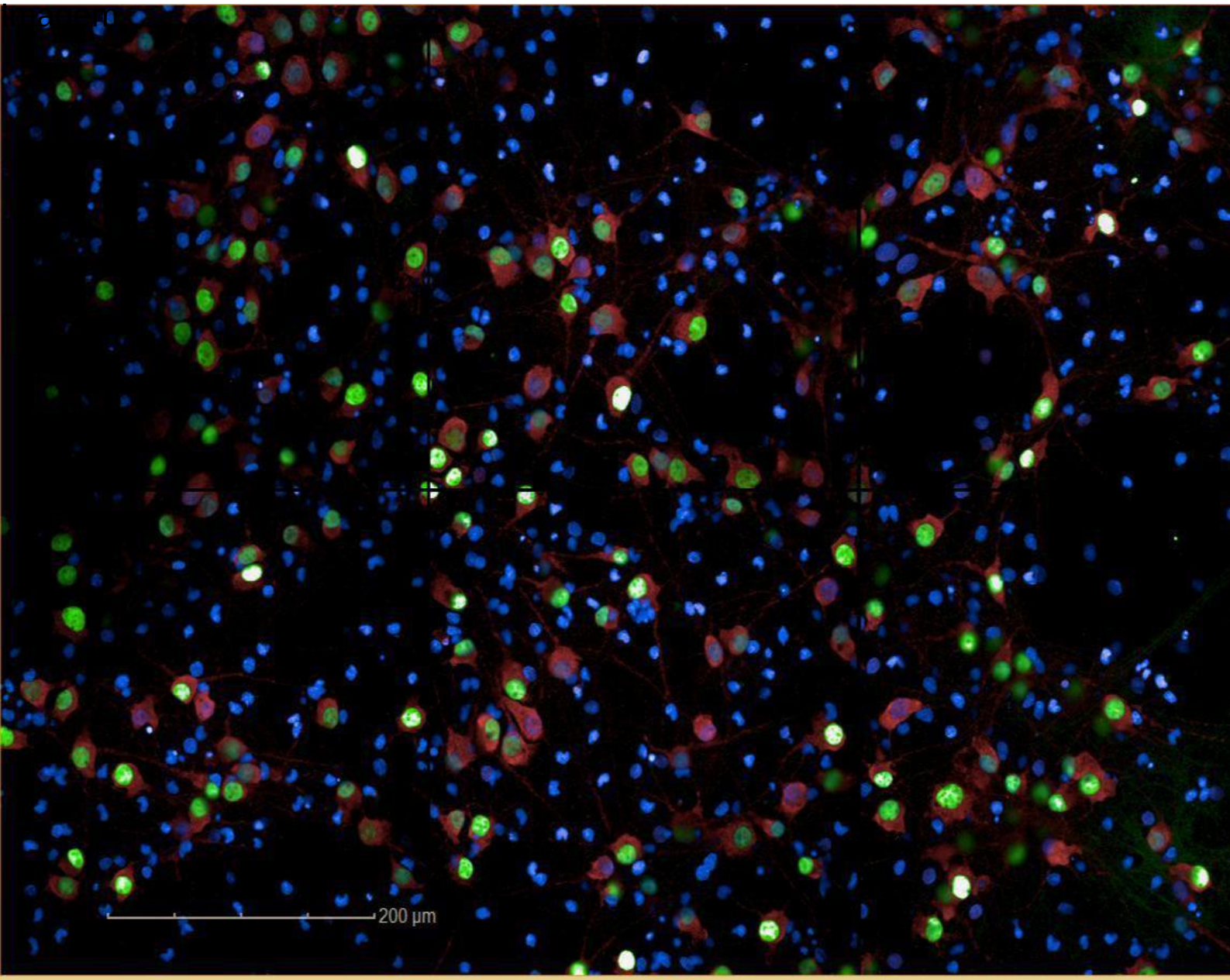

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Dissertação de Mestrado em Biologia Celular e Molecular

Junho 2017

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Universidade de Coimbra

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Assaying synaptic function using genetically-encoded optical reporters in the context of Alzheimer's disease

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pelo Doutor Nachiket Kashikar (Departamento de Neurociências, Janssen Pharmaceutica) e pelo Professor Doutor Carlos Duarte (Departamento de Ciências da Vida, Universidade de Coimbra) e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.

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List of abbreviations:

AAV – Adeno-associated virus
AD – Alzheimer's Disease
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP – Action Potential (electrical stimulation pulse)
APOE4 – Apolipoprotein
APP – Amyloid Precursor Protein
BACE-1 - β-secretase named beta-site APP cleaving enzyme 1
BSA – Bovine serum albumin
CaM – Calmodulin
ChR2 (TC) – Channelrhodopsin-2 T159C mutant
CNS – Central Nervous System
cp – circularly permuted
CS - Coverslip
DIV – Days in vitro
DMSO – Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
EF hands – Calcium-binding motifs composed of two helixes (E and F) joined by a loop
EPSPs - excitatory postsynaptic potentials
ER- endoplasmic reticulum
GABA - Gamma-AminoButyric Acid
GECI – Genetically-encoded calcium indicators
GFP – Green Fluorescent Protein
GPCRs - G protein-coupled receptors
HEBS - 2xHEPES buffered saline solution
IP3 - inositol triphosphate
LED - light-emitting diode
LTD - Long Term Depression
LTP – Long Term Potentiation
mGluRs – metabotropic Glutamate Receptors
MOI – Multiplicity of Infection
NBM – Neurobasal medium
NES – normal extracellular solution
NMDA – N-methyl-D-aspartate
PAM – Positive AMPA Modulator
PBS – Phosphate-buffered saline
PDL - Poly-D-Lysine
PFA – Paraformaldehyde
PLC – Phospholipase C
PSD – Post Synaptic Density
RFP – Red Fluorescent Protein
ROI – Region of Interest
RyRs - ryanodine receptors
SNR – Signal-to-noise ratio
TE buffer – Buffer containing Tris and EDTA
VGCCs - voltage-gated calcium channels

Abstract

Alzheimer's disease (AD) is a devastating disease affecting mostly the elderly population[1]. Current studies show that importantly, not amyloid or Tau pathology *per se*, but synapse dysfunction/loss and the consequent loss of connectivity between brain regions correlates well and underlie the cognitive and memory symptoms of AD[2].

Since synaptic loss is a reversible process and can be detectable in the early phases of the disease progression, it is important to develop the tools necessary for assaying synaptic function in the context of AD pathology.

In this project, we used genetically-encoded calcium indicators (GECIs) to assay synaptic function. Changes in pre and post-synaptic Ca^{2+} were monitored using SyGCaMP6f, SyjRCaMP1b, and SyjRGECO1a as pre-synaptic calcium reporters and actin-GCaMP6f as post-synaptic calcium reporter. Neuronal excitability was evaluated by characterizing a recently published nuclear calcium reporter - H2B-GCaMP6f. Neuronal excitability can be considered as a combination of intrinsic membrane properties - including membrane resting potential and input resistance - with synaptic plasticity events[3,4]. Organic dyes such as Fluo-4 or Cal-520 can be used to measure neuronal excitability, however fluorophores do not show a sensitive voltage-dependent signal with a single cell resolution due to its diffusion along the entire neuron[5]. To overcome this drawback we developed a new construct - AAV6:hSynapsin1:H2B-GCaMP6f -in which the fastest green genetically-encoded calcium reporter - GCaMP6f[6] - was fused to a H2B sequence[7,8]. H2B-GCaMP6f was characterized as a nuclear calcium reporter as well as characterized its utility of this reporter in preliminary pharmacology assays by testing three different compounds that modulate neuronal excitability – a KV1.3 channel blocker[9], LY450108[10] (AMPA PAM) and a confidential compound from a phenotypic screening effort within J&J, called Compound X. Pharmacological tests were performed in primary neuronal cultures of rat hippocampus in two conditions - presence of 0.01 mM NBQX (AMPA antagonist) and 0.05 mM D-AP5 (NMDA antagonist), and absence of these synaptic blockers. Beside H2B-GCaMP6f characterization, the dynamic range of all the pre-synaptic reporters mentioned above was characterized by delivering trains of different number of electrical stimuli at 20Hz. Multiplex imaging using SyjRGECO1a and H2B-GCaMP6f was also performed to analyze nuclear and synaptic response simultaneously.

Combining a fully characterized nuclear reporter -H2B-GCaMP6f- with green and red shifted variants of sensitive calcium reporters, we are developing tools to a better understanding of neuronal excitability and synaptic function that can be further used during phenotypic or target-based drug screening efforts in the context of Alzheimer's disease or other neurological diseases.

Keywords: Genetically-encoded calcium reporters; neuronal excitability; Alzheimer's disease.

Resumo

A doença de Alzheimer é uma doença que afecta maioritariamente a população sénior[2]. Estudos recentes comprovam que não é a patologia das proteínas amilóide β e Tau *per se* mas sim a disfunção e/ou perda de sinapses, e consequente perda de conectividade entre regiões do cérebro, que originam os sintomas relacionados com a memória e os processos cognitivos na doença de Alzheimer[3].

Sendo que a disfunção sináptica é um processo reversível que pode ser detectado em fases iniciais da doença de Alzheimer, é importante desenvolver ferramentas necessárias para o estudo da função sináptica no contexto desta doença.
Para tal, neste projecto foram usados indicadores de cálcio geneticamente codificados denominados repórteres. Mudanças na concentração de cálcio na região pré sináptica foram estudadas usando repórteres como SyGCaMP6f, SyjRCaMP1b e SyjRGECO1a e na região pós-sináptica, usando reporters como Actina-GCaMP6f. A excitabilidade neuronal foi estudada através da caraterização de um reporter recentemente publicado que é direcionado para o núcleo denominado H2B-GCaMP6f. A excitabilidade neuronal advém de uma combinação de propriedades intrínsecas da membrana celular (onde se inclui o potencial de membrana em repouso e a resistência da membrana) com a plasticidade sináptica[4,5]. Fluoróforos orgânicos como Fluo-4 ou Cal-520 podem ser usados para medir a excitabilidade neuronal, contudo esses fluoróforos não exibem um sinal suficientemente sensível a mudanças de voltagem na membrana porque são expressos e difusos ao longo do neurónio[6]. Desta forma, desenvolveu-se um *construct* - AAV6:hSynapsin1:H2B-GCaMP6f – em que o repórter mais sensível desenvolvido até hoje – GCaMP6f – é ligado por engenharia genética a uma sequência da histona H2B[7, 8]. H2B-GCaMP6f foi caracterizado como um repórter nuclear sensível ao cálcio assim como a sua utilidade em testes preliminares de farmacologia, tendo sido usado três moduladores de excitabilidade neuronal: um inibidor de canais de potássio depedentes da voltagem (KV1.3 blocker) [9], um modulador positivo dos receptors AMPA (AMPA PAM) - LY450108[10] – e um composto confidencial vindo de uma análise fenotipica da J&J,denominado Composto X. Testes farmacológicos foram realizados em culturas neuronais primárias de hipocampo de rato em duas condições experimentais diferentes: na presença de 0.01 mM NBQX (antagonista dos receptors AMPA) e 0.05 mM D-AP5 (antagonista dos receptores NMDA) e na ausência deste inibidores sinápticos. Para além da caracterização de H2B-GCaMP6f, a dinâmica de todos os repórteres pré sinápticos mencionados acima foi também caracterizada através de estimulação eléctrica de sequências com diferentes números de estímulos eléctricos a 20Hz. Gravações em tempo real foram também efectuadas usando SyjRGECO1a e H2B-GCaMP6f para comparar respostas sinápticas e nucleares simultaneamente.

Combinando um repórter nuclear integralmente caracterizado, como o H2B-GCaMP6f, com diferentes repórteres sinápticos emitindo fluorescência com o comprimento de onda na zona verde e vermelha do espectro, é possível desenvolver ferramentas para uma melhor compreensão da excitabilidade neuronal e função sináptica, que futuramente poderá ser

usada para análises fenótipicas ou dirigidas de compostos no contexto da doença de Alzheimer ou outras doenças neurológicas.

Palavras-chave: Indicadores de cálcio geneticamente codificados; excitabilidade neuronal; Doença de Alzheimer.

1. Introduction

Synaptic transmission

Synapses are the way neurons can communicate with each other by electrical and/or chemical responses. A normal synaptic transmission such as a glutamatergic one occurs when an action potential arrives at the presynaptic terminal and opens voltage sensitive calcium channels, allowing a brief influx of calcium into the presynaptic terminal. This influx triggers neurotransmitter-containing vesicle fusion leading to neurotransmitter release into the synaptic cleft. Then the neurotransmitter interacts with postsynaptic receptors to activate downstream signaling cascades[11].

Synaptic activity reporters

All the synaptic transmission steps can be measured with exquisite spatiotemporal precision using fluorescent reporters. There are several reporters used to assess synaptic functions, such as genetically encoded calcium indicators to investigate pre-and postsynaptic calcium influx, synaptic function sensors such as iGluSnFR to study glutamate release, SypHy for vesicle fusion, and voltage sensitive reporters as a measure of change in membrane voltage.

Genetically Encoded Calcium Indicators (GECI)

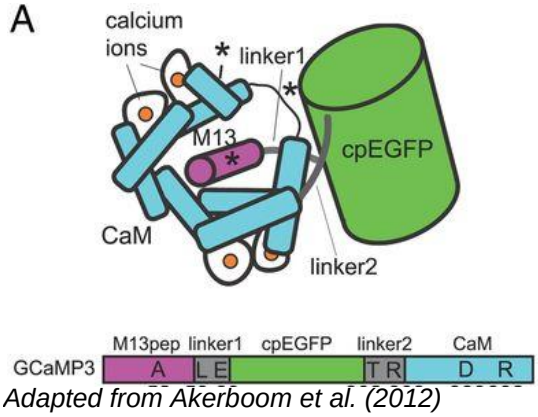
Calcium is an important secondary messenger in synaptic transmission because it is responsible for triggering the fusion of vesicles containing neurotransmitter in the presynaptic membrane. When an action potential reaches the axon terminal a large and fast influx of calcium flows into the cytoplasm through voltage sensitive Ca^{2+} channels. It is this peak of intracellular calcium that leads to the release of neurotransmitters to the synaptic cleft, so in this way the increase of intracellular Ca^{2+} is associated with an action potential arrival and many researchers started to study synaptic function by tracing these calcium transients. Although the techniques for this approach were not compatible with chronic measurements due to the invasiveness and damage of the tissue induced by bulk or intracellular loading of calcium synthetic indicators[12].

Taking into account these drawbacks, Genetically Encoded Calcium Indicators (GECIs) were developed, which are chimeric fluorescent proteins that can target precise structures such as synapses, dendrites and axons, and also target specific populations of neurons, throughout a noninvasive approach [13]. This new technique allows chronic *in vivo* measurements of calcium transients in living cells. These chimeric fluorescent proteins were added to a calcium-binding domain, either Calmodulin or Troponin C, which induces a conformational change when calcium binds. This conformational change causes fluorescence or color changes meaning it is then possible to image neural activity *in vivo*[14]. It is noteworthy that there is still no perfect standard GECI because different applications requires different affinities and expression of the reporter [12]. For instance, a high affinity sensor produces large signal changes with small calcium transients however it reaches the saturation point more easily [15], on the other hand a poor affinity sensor presents its maximal signal changes at saturated calcium concentrations

though it is better to measure high calcium transients as those of synaptic receptors[12]. Regarding GECI expression it is known that low expression leads to poor SNR and it then becomes more difficult to visualize the positive cells. Although, high expression leads not only to an excessive brightness that consequently leads to a higher SNR and then to a decrease on signal changes but also can modify the calcium homeostasis. These parameters should be optimized before each application in order to balance cytotoxicity with good signal.[16]
In order to optimize GECIs, a computational design was run in order to diminish interferences with endogenous neuronal proteins so it tested a direct substitution of calmodulin for a skeletal binding protein – Troponin C - which is not expressed as much in neurons so it is less recognized in the neuronal environment which reduces the endogenous interferences[17]. Not only was the interferences problem optimized, but there were also improvements on brightness baseline, dynamic range and calcium affinity.[12]

Kinetic properties of a GECI

K_{off} and K_{on} are constants that clarify about the kinetic properties of a calcium reporter and reflect kinetics events as 1. Calcium association/disassociation rate to the EF hand of the calmodulin, 2. the conformational changes induced after the binding of calcium to the calmodulin EF hands and 3. the interaction between calmodulin and M13 peptide[18]. Therefore, calcium sensitive dyes exhibits less conformational changes and interactional steps from calcium binding to fluorescence emission[18,19] and that is why it is not surprising that both on and off kinetic reactions are slower in GECIs responses than in calcium- sensitive dyes responses.
The development of faster GECIs is a challenge due to the fact that on and off kinetics, affinities and maximal fluorescence are all linked to one another by the equation $K_d = k_{off}/k_{on}$, so in order to optimize one parameter some advantages of the others could be lost[19]. The focus has been on the shortening of the sequential conformational changes steps more specifically on EF hand loops' chelating calcium residues or mutagenesis in the binding interface of calmodulin with M13 peptide[19] (Figure 1.1). It was also shown that calcium affinities and kinetics of a reporter are closely and reciprocally related[19].



Adapted from Akerboom et al. (2012)

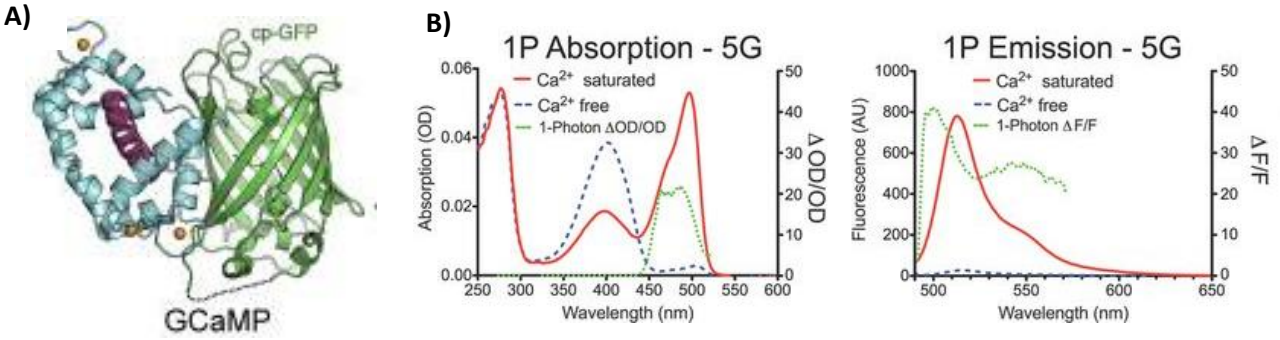
Figure 1.1 – Example of engineering sites represented by "*" in the schematic structure of GCaMP3. In the figure are represented the following sites used for GCaMP3 directed mutagenesis: 1.the cpGFP/CaM proto-interface, 2.mutations on M13 peptide and 3. mutations near the third Ca2+-binding site of CaM. Even though it is not

shown, M13peptide-cpGFP and cpGFP-CaM linkers were also engineering targets. The engineering sites mentioned above were involved in the update of GCaMP3 to GCaMP5 variants[20].

GFP-based GECIs

GFP-based GECIs are calcium indicators that have an excitation spectrum that peaks at 475-495nm and emission spectrum at 520-560nm[21] (Figure1.2 B).

The most known calcium reporter is GCaMP which is used in a single fluorescent protein named cpGFP due to its circularly permuted enhanced GFP signal. There were several improvements on this reporter and nowadays the best reporter for detecting single action potentials in pyramidal neurons is GCaMP6 which has greater protein stability, dynamic range, calcium affinity and brightness than the previous ones[22].

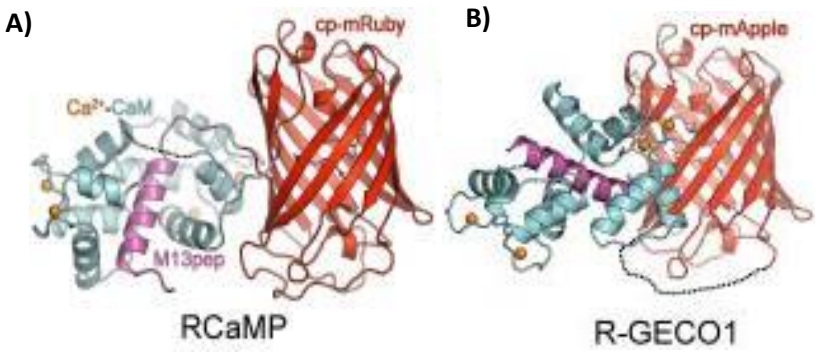


Adapted from Akerboom J et al. (2013) and Akerboom J et al. (2012)

Figure 1.2- Crystal structure of GCaMP in the presence of calcium (left) and one-photon excitation/emission range of GCaMP5G.A) A circularly permuted GFP (cp-GFP) is linked to CaM-M13 complex (CaM in blue and M13 peptide in purple) that will modify the chromophore environment inside the GFP β-barrel (in green) once calcium (in yellow) binds the complex. B) one-photon excitation and emission spectra of GCaMP5G in Ca-free (dashed lines) and Ca-saturated (red lines) conditions.

RFP-based GECIs

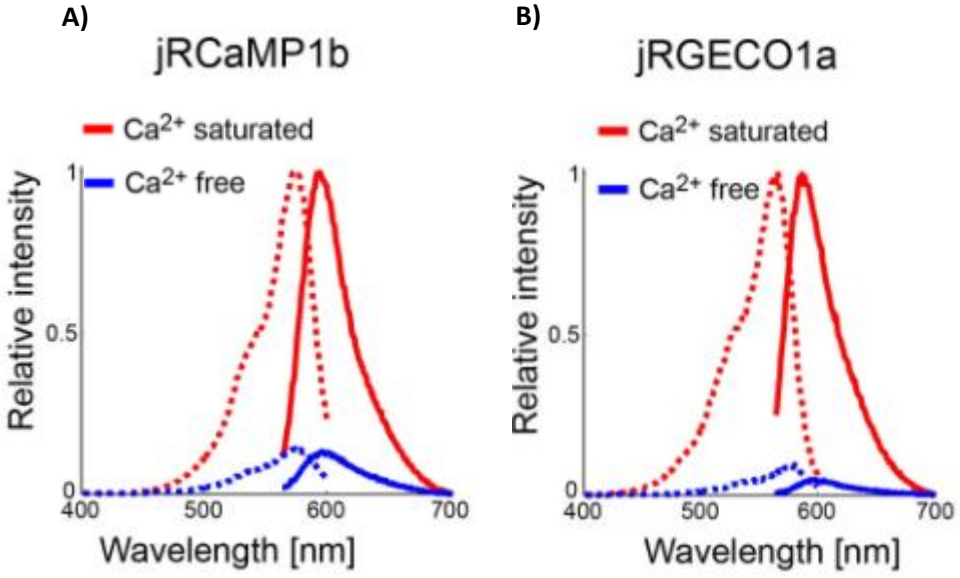
RCaMPs -which share the same architecture as GCaMP sensors but instead of a circularly permuted green fluorescent protein (GFP) they have a circularly permuted red fluorescent proteins (RFPs)[23] – have in its conformation the calmodulin (CaM) and the M13 peptide that interact with CaM. As in GCaMP indicators, in the presence of calcium there is a conformational change in CaM which will link to M13 forming a complex that will modify the chromophore environment inside the RFP β-barrel[23]. This alteration will lead to an increase of the RFP brightness.



Adapted from Akerboom J et al. (2013)

Figure 1.3- Crystal structure of RCaMP and R-GECO1a in the presence of calcium. A) A circularly permuted mRuby (cp-mRuby) is linked to CaM-M13 complex (CaM in blue and M13 peptide in purple) that will modify the chromophore environment inside the RFP β-barrel (in red) once calcium (in yellow) binds the complex. **B)** A circularly permuted mApple (cp-mApple) is linked to CaM-M13 complex (CaM in blue and M13 peptide in purple) that will modify the chromophore environment inside the RFP β-barrel (in red) once calcium (in yellow) binds the complex.

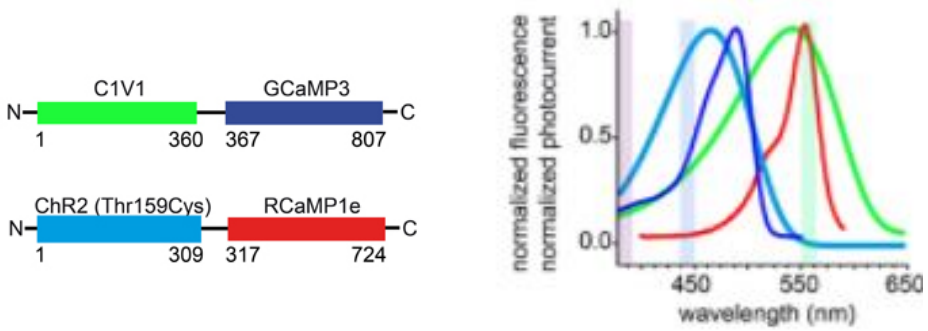
Although red GECIs were not so common for detecting and quantifying synaptic activity as GCaMP6f sensors, these reporters have now room to be improved and variants as jRCaMP1b and jRGECO1a started to appear[23] (Figure 1.3). Their excitation and emission spectra are shown in Figure 1.4.



Adapted from Dana et al. (2016)

Figure 1.4 – Excitation and emission range of jRCaMP1b and jRGECO1a in Ca-free (blue lines) and Ca-saturated (red lines) conditions. A) jRCaMP1b excitation (dashed lines) and emission (solid lines) range spectra. B) jRGECO1a excitation (dashed lines) and emission (solid lines) range spectra.

These new spectrally red-shifted variants are now facilitating not only multiplex imaging together with GFP-based reporters but also *in vivo* imaging since most of transgenic animals contains a GFP marker. Importantly, the improvement of red shifted variants of GECIs allows the combination of neuronal calcium imaging and the use of optogenetics too[23,24].To avoid overlapping the broad excitation spectrum of opsins[25] with GECIs' excitation spectrum, either the calcium indicator or the channelrhodopsin must be spectrally distinct. Since the most red- shifted variant of channelrhodopsin[26] spectrum still overlaps significantly GECIs' excitation spectrum, the perfect combination would be a red shifted variant of GECI with blue light activated ChR2[24]. Multi-color experiments using a combination of channelrhodopsin-2 (ChR2) to activate neuronal activity together and RCaMP to measure calcium signaling are described in Akerboom et al. (2013)[24]. Constructs used in those combined experiments are descried in Figure 1.5 as well as the respective excitation spectra.



Adapted from Akerboom J et al. (2013)

Figure 1.5 – Constructs used in Akerboom et al. (2013) for multiplex imaging and respective excitation spectra. On the left, schematic of C_1V_1 fused to GCaMP3 on top and ChR2 (TC) fused to RCaMP1e on the bottom. On the right, excitation spectra of the effectors ChR2(TC) and C_1V_1 and the calcium reporters RCaMP1e and GCaMP3 (colors correspond to the constrcuts on the right).

GECIs targeted to specific structures

- **Pre-synaptic GECIs**
    - **SyGCaMP6f**

All GECIs explained above were cytoplasmic calcium indicators, nevertheless there are GCaMPs that can track synaptic vesicles in pre-synaptic compartments due to its fusion to synaptophysin on its cytoplasmic side[27]. Synaptophysin is a synaptic vesicle's transmembrane protein with very low expression on the cell surface which allows us to trace a neurotransmitter-containing synaptic vesicle in a terminal axon *in vivo*[27,28]. These reporters are named SyGCaMPs. SyGCaMPs have two advantages over the cytoplasmic GECIs which

are not only the instant identification of synapses' location even at rest, but also the saturation and linearity of the response among different spike is enhanced. Saturation and linearity show better results in SyGCaMPs due to their localization in a presynaptic compartment near the high density of voltage dependent calcium channels where the calcium transients are brief instead of having changes in bulk calcium concentrations[27]. To get the most sensitive pre-synaptic reporter, a GCaMP6f - the most recent and improved GCaMP[22]–was fused to a synaptophysin forming a reporter called SyGCaMP6f.

- **SyjRCaMP1b**

jRCaMP1b is a mRuby-based red reporter[24,29], and it is a variant of RCaMP1h. RCaMP1h is one output of the first generation of RCaMP1 structure-guided mutagenesis with mutations focused on the edges between the RFP and CaM, between the latter and M13 and in CaM itself, in order to improve the sensitivity of the reporter. Then, from RCaMP1h was done a second round of beneficial random mutagenesis in which came out variants as jRCaMP1b – a high sensitive mRuby-based red spectral variant GECI [23].
It showed equivalent absorption and emission spectra as well as construction comparing to the first generation parental reporter- RCaMP1 - however jRCaMP1b differs significantly in sensitivity and kinetics[23].
SyjRCaMP1b is a fusion of RCaMP1b to presynaptic protein named synaptophysin, performed by molecular biology engineering

- **SyjRGECO1a**

jRGECO1a is a mApple-based[30] reporter which is an improved variant from RGECO1[31]. As it was described above for jRCaMP1b, RGECO also went through two rounds structure-guided and random mutagenesis and then a neuron-based screening. jRGECO1a is the final output being a high sensitive mApple-based reporter [23].
This high sensitive mApple-based GECI exhibits equivalent performance as GCaMP6f indicators, although also exhibits photoswitching when it is illuminated with blue light, which makes it less compatible for experiments in combination with optogenetics[23].
For presynaptic studies, jRGECO1a was fused to synaptophysin by molecular biology engineering and became SyjRGECO1a. SyRGECO – not the variant RGECO1a- was used before for functional imaging in the zebrafish retinotectal system[32].

- **Post-synaptic GECIs**

- **PSD95-GCaMPs**

To assay calcium changes in post synaptic activity the most abundant protein in post synaptic density – PSD95- is targeted. As SyGCaMP, PSD95-GCaMP are closed to the receptors which allow calcium to pass through so the calcium transients are also brief [28,33]. With this reporter it is possible to monitor calcium entry through NMDA receptors[34].

PSD95-GCaMP that emit green fluorescence due to GFP in GCaMP can also be used in a dual-color imaging system when combined with a reporter of presynaptic activity fused with a vesicle transmembrane protein- synaptophysin- for instance SypHyTomato reporters which produces red fluorescence, with the aim of determining temporal correlation between presynaptic vesicle fusion and postsynaptic calcium changes [34].

- **Actin-GCaMP6f**

Actin is fundamental not only for neuronal structure but is also important on muscular contraction, together with myosin, in skeletal cells[35]. In addition, actin is present mostly at the spines as well as at the edges of the axons[36,37]. One theory for being most concentrated in these compartments could be the necessity of alterations of spine shape as a response to a more prolonged stimulus as well as for stabilization of Post Synaptic Density (PSD)[37]. Even though actin is spread along the neuron yet on a lower concentration than spines, it is possible to target for dendritic spines expression as it was done before with a GCaMP2-chicken B actin fusion for targeting subcellular compartments in pyramidal cell in hippocampal slice cultures in mice [33]. This targeted GECI were a plasmid with chicken β actin protein fused to the C-terminus of GCaMP2 with the linker sequence GGR[33]. However, the authors referred two drawbacks related to GCaMP2-actin and GCaMP2 imaging. The first was that it was difficult to locate spines because both reporters – GCaMP2 and GCaMP2-actin - were quite dim. Secondly they mentioned the high photobleaching of SyGCaMP2 comparing to cytoplasmic GCaMP2, which is a normal issue when targeted GECIs are being imaged, meaning that the time to recover fluorescence of actin-GCaMP2 was slower than the freely diffusible cytosolic GECIs[33]. Beside the referred drawbacks, actin is mostly present at post synaptic spines and it is also possible to target it for dendritic spines[33] so it is a good candidate to be a genetically encoded fluorescent reporter for tracking synapse activity on post synaptic sites. To overcome the drawbacks mentioned above, it was linked the same actin sequence from Mao *et al.* (2008)[33] to a GCaMP6f, since it is the most recent GCaMP, and the result was Actin GCaMP6f.

- **Nuclear GECI**

- **H2B-GCaMP6f**

Knowing that synaptic inputs have influence on nuclear functions[38] including genomic[39] and nongenomic activities[40], nuclear genetically encoded calcium reporters were design to a better understanding and visualization of nuclear excitability[7].
To study nuclear calcium responses, the most recent published nuclear genetically encoded reporter is named H2B-GCaMP6f[7]. Previously such a construct using GCaMP6f was used in zebrafish to monitor pan-neuronal activity under a light-sheet microscope, while zebrafish was performing simple tasks in a virtual reality environment. We decided to follow this approach and characterize it further in primary neuronal cultures to explore its utility in drug screening. H2B is one of the four core histones – together with H2A, H3 and H4 - that constitutes a nucleosome[41,42]. Nucleosomes are fundamental nuclear structures that maintain and control

the stability of chromatin by a tightly controlled organization of histones, which are commonly used to fuse nuclear tags for nuclear localization[7,41,42].
Having a nuclear calcium reporter as H2B-GCaMP6f makes possible a whole-brain imaging for neuronal circuits studies[7] as well as individual nuclear calcium responses as a neuronal excitability measurements[7,8].

Neuronal excitability

Neuronal excitability is considered stable throughout an animal life except in times of development or degeneration due to aging or pathology[4]. This stability is kept due to a combination of synaptic plasticity and intrinsic membrane properties[4]. To study intrinsic membrane properties there are two keys parameters to analyze – membrane resting potential and input resistance[5]. Changes in intrinsic membrane properties that affect neuronal discharge rate or pattern were reviewed by Beraneck and Idoux (2012) on a study about vestibular nucleus neurons' excitability[43]. Similar alterations occur in other pathologies of CNS such as epilepsy in which voltage-dependent potassium channels in pyramidal neurons are linked to a cell being more sensitive of small voltage fluctuation as well as the hyperpolarization of the resting membrane potential[4]. Not only are the potassium voltage dependent channels involved in changes on intrinsic neuronal excitability but also the amount of voltage dependent sodium channels were shown to be involved in the reduction of the required threshold to induce an action potential from dorsal root ganglion neurons in chronic pain[4]. Beside the changes described above about spike threshold and action potential shape, there are also other experience-dependent changes in which occurs the alteration of neuronal firing pattern such as in the number and consistency of spikes over time[4]. This last type of changes in intrinsic neuronal firing mode were also reported in studies related to CNS pathologies[4]. In addition it was shown that extracellular calcium also plays a role on neuronal excitability via activation of UNC79-UNC80-NALCN complex in a G-protein-dependent manner[44]. It is important to understand how the firing pattern of an individual neuron can be altered not only to understand more underlying features of some neuronal diseases but also as a target to their therapeutic[4].
One of the mechanisms to study alterations in intrinsic neuronal excitability is pharmacology in which is possible to pinpoint the mechanisms of action behind neuronal excitability by the compound-inducible changes[5]. As explained above it is important to study synaptic plasticity and intrinsic membrane properties to understand neuronal excitability. In order to study synaptic plasticity, the experiments have to be focused on the action of synaptic receptors such as NMDA and AMPA[45]. Although NMDA receptors are located on synapses, it was shown that they play a permissive role at inducing nuclear calcium signaling, not directly by calcium flux but by the generation of a consequent action potential[38]. However, to study specifically the intrinsic neuronal excitability it is required to block the synaptic receptors so there are no neurotransmitters inputs[43,46] and the focus can be the electroresponsive properties of single neurons[43].
There are several compounds that modulate neuronal excitability, one of them is KV1.3 channel blocker, which leads to membrane depolarization[9]; another neuronal excitability

modulator known from the literature is LY450108 which is a Positive allosteric Modulator of AMPA receptors involved in studies regarding synaptic plasticity and memory processes[10].
Two well-known compounds used to block AMPA and NMDA receptors activity are glutamate receptor antagonists NBQX as AMPAR antagonist and AP5 as NMDAR antagonist which are used together in hippocampal studies to block the activity of both receptors [47]. In addition, It was shown that the blockade of spikes induced by NMDAR antagonist reduce the entry of calcium through VGCC[38].
Calcium signaling is an important mechanism that controls survival, proliferation, gene expression, between other important cellular and molecular functions[48]. It is known that nuclear calcium signaling are closely related to genetic and molecular changes and that cytoplasmic Ca+ activity does not play the same role as nuclear Ca2+ activity in gene expression control[38]. The mechanisms behind synapses –nucleus communication is not fully understood, yet some theories are being proposed[38,39,49,44,50].
It is believed that the calcium-based communication between synapses and nucleus is centered on two mechanisms. The first mechanism started with the glutamatergic activation of NMDA, Kainate and AMPA receptors by neurotransmitter's binding which will lead to induction of excitatory postsynaptic potentials (EPSPs)[49,50]. These EPSPs will trigger an action potential that will back propagate along the membrane leading to membrane depolarization and consequent activation of voltage-gated calcium channels (VGCCs)[39,49,50]. Not only membrane depolarization can lead to an increase of nuclear calcium concentration but also endoplasmic reticulum (ER) or Golgi apparatus play a role on it by releasing calcium via ryanodine receptors (RyRs) or inositol triphosphate (IP3) receptors[39,49]. The second mechanism is related to G protein-coupled receptors (GPCRs) and its downstream signaling. Once glutamate binds to mGluRs which are coupled to PLC, IP3 is produced. IP3 as well as calcium can stimulate IP3 receptors on ER membrane which will lead to more release of calcium creating a propagating intracellular calcium wave that started from dendrites and it ends on boosting nuclear calcium signaling via IP3 nuclear receptors and nuclear pores complexes[39,49,50]. ER membrane plays an important role in the transmission of synaptic evoked currents from the dendrites to the nucleus and vice versa[50].Regarding ER, its nuclear-dendritic location together with plasma membrane conductivity facilitates spines-evoked currents propagation as well as calcium amplification across and along the ER membrane from synapses to nucleus[49,50].
Once synaptic blockers are present the only inputs that could trigger an action potential would be a summation of several EPSPs or a strong external stimulus[39,50]. When excitatory input is not strong enough or the summation of EPSPs do not reach the action potential threshold then depolarization does not occur and consequently somatic L-VGCCs would not open[39]. On the other hand, if the synaptic input is strong enough to reach action potential threshold, it will easily propagate and originate action potential at the axonal initial segment and then it will back propagate to the soma[39]. Once electrical inputs arrive near to soma it will open somatic and perisomatic L-VGCCs and consequently calcium will cross the nuclear envelope to get in to the nucleus by its concentration gradient[50]. Calcium entry into the nucleus will induce several important calcium dependent cascades[39,50]

Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a devastating disease affecting mostly the elderly population. It is a type of dementia characterized by two definitive proteineous brain lesions: Aβ plaques and neurofibrillary tangles, which are formed through the deposition of amyloid beta and hyperphosphosrilated tau protein, respectively[2].

Besides the most common type of this neurodegenerative disease is the sporadic AD, there is still 5% of the total affected patients who have early-onset familial AD. This rare type of AD is described by an autonucleusl dominant mutation in the PSEN1/2 and amyloid precursor protein (APP) genes. The PSEN1/2 gene is involved in the y-secretase complex which it is responsible for the APP cleavage and subsequent formation of the toxic Aβ. Both mutations are associated with an increase of Aβ and failure in its clearance, leading to a toxic Aβ deposit and its early accumulation [51–54]. This is the literature background behind this familial type of AD however the mechanism for the sporadic one is not so fully understood[55]

Molecular and structural Features in AD

One of the major visible feature of Alzheimer disease is the massive neuronal loss and subsequent atrophy of specific areas of the brain which leads to a decrease of brain weight of almost a third [2,3]. The most affected structures are the limbic and specific cortical areas – frontal, parietal and temporal association cortices[2]. Noteworthy that cortex areas such as primary and sensory ones are not affected as much as the other ones even in advanced AD stage and appear almost normal in neural imaging studies[2,3]. Although the association cortices are more susceptible to degeneration during ageing [56].

There are also small structural changes in neurons such as in axons and dendrites' morphology and trajectories as well as changes in post synaptic structures which could affect neuronal transmission[2].

Regarding the molecular mechanisms that lead to Aβ accumulation in familial AD there are several evidences supporting the most accepted theory – "amyloid theory" [51–53]. This theory is based on *in vivo* proteolytic cleavage of the APP firstly by a β-secretase named beta-site APP cleaving enzyme 1 (BACE-1) and then by a γ -secretase, generating Aβ peptides , being the $A\beta_{1-42}$ the neurotoxic ones, and constitutes the end of the amyloidogenic pathway. On the other hand there is a pathway which produces p3 fragments instead of Aβ ones due to the two cleavages that APP pass through in this non amyloidogenic pathway- first is cleaved by α-secretase and then a γ-secretase [57–60]. Nonetheless if it occurs a dysregulation of this two processing APP pathways balance as well as an impairment of mitochondrial function or even a deficiency on Aβ clearance mechanisms it is induced the release of Aβ peptides including the neurotoxic ones to extracellular environment as well as the promotion of its aggregation into senile plaques [61–63].

Synaptic Dysfunction in AD

Synaptic impairments are associated with several mechanisms related to the presence of soluble amyloid on the synapse and its interactions as well as the presence of tau in post synaptic density and its potential link with Aβ[3] (Figure 1.6).

There are several studies suggesting that there is indeed an impairment of neural system function however the causes of this dysfunction are not still fully understood yet it is likely that amyloid and tau play an important role [64]. In 2001, Masliah observed a significant loss of synaptophysin, a presynaptic marker of vesicles' fusion [65] and during the last years it was found in mouse models as well as in post mortem human tissue that around dense-core amyloid plaques the synapses were lost, however the same event do not occurs around diffuse plaques, suggesting that neuritic plaques release molecules which can be toxic to synapse function[66,67].

There are several others pathogenic factors such as other APP metabolites, tau, apoE4, α-synuclein, vascular alterations as well as glial responses, inflammation, oxidative stress and epigenetic and environmental determinants that also play a copathogenic important role in AD specially in sporadic form of the disease[68].

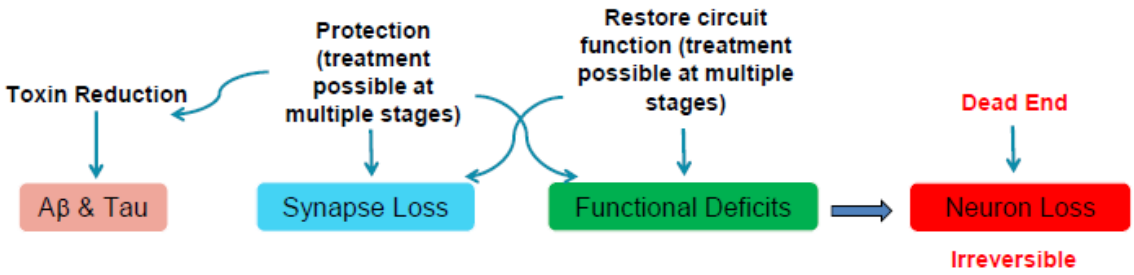


Figure 1.6- Sequential events of Alzheimer's disease progression that lead to an irreversible loss of neurons. On the top, for each reversible stage of progression, it is shown how therapeutics can act towards the reversibility of the event.

Neuronal plasticity

Neuronal plasticity is a crucial compensation mechanism for lost or disrupted circuits, and some of them include the cost of network failure for a cell survival [69] yet others can enhanced neuronal plasticity to help the remaining neural circuits by adjusting some neuronal processes in order to overcoming the neuronal weaknesses. Improvement of neuronal plasticity helps to prevent the inexorable neuronal loss that occurs in AD as well as increase the overall network performance of circuits. Electrodes are the most common technique to stimulate synaptic function and plasticity[70] however environmental and molecular stimulation was proved to increase synaptic plasticity enough to delay and diminish neurologic changes in AD mouse models [71]. The latter can be assayed with the basis of the molecular and signaling changes that amyloid beta does in synapses by binding various cell-surface molecules which include neurotransmitter receptors as mentioned above[70].

There are several drugs within the purpose of binding neurotransmitter receptors and enhance their signaling, however the blocking of signaling pathways are often related to a downstream abnormal protein assembly so the administration of these drugs cannot overcome upstream modulation [70]. One of the AD affected signaling pathways is the glutamatergic one as well as the calcium signaling[69,72,73].

Aim of the work

The aim of this project is to characterize and compare genetically-encoded calcium reporters targeted to different structures of the neuron as: 1. pre-synaptic compartment using SyjRCaMP1b, SyjRGECO1a and SyGCaMP6f, 2.post-synaptic compartment testing Actin-GCaMP6f and 3. nucleus using H2B-GCaMP6f. Intrinsic neuronal excitability will be measured with the development and characterization of a nuclear calcium reporter (H2B-GCaMP6f) while pre-synaptic activity will be measured by tracing calcium transients using GFP and RFP-based calcium reporters fused with synaptophysin. Within the development and characterization of different sensitive tools to study synaptic function in primary hippocampal cultures from wild type animals, the goal is to apply these tools on further phenotypic or target-based drug screening efforts in the context of Alzheimer's disease or other neurological diseases.

2. Methods

Primary Neuronal Cultures

Hippocampal cultures dissociated from Wistar Charles River E19 pups were plated in 12 well plates in poly-d-lysine coated coverslips type GG-15-PDL - ø 15 mm from Neurovitro**.** Neuronal cultures were incubated first with 2 ml of NeuroBasal Medium (NBM) without Phenol Red and High concentration of glutamax (Medium A, described below) but then the medium changes were performed twice a week by taking half of the previous one and adding half of new NBM but with low concentration of glutamax (Medium B, described below). For SyjRCaM1b's experiments, the cell density was 60000 cells per well while for SyjRGECO1a, SyGCaMP6f and H2B-GCaMP6f's experiments the cell density was 130000 cells per well.
Medium A composition was 500 ml of Neurobasal Medium without Phenol Red (Invitrogen 12348017); 10 ml B27 (Invitrogen 17504044); 5 ml Glutamax (Gibco 35050-038).
The medium B used to medium changes was composed for 500 ml Neurobasal Medium without Phenol Red (Invitrogen 12348017); 10 ml B27 (Invitrogen 17504044); 1,25 ml Glutamax (Gibco 35050-038) and 7 ml Penicillin-Streptomycin (SIGMA P4333).

Transductions

Circular map and sequence of SyjRCaMP1b, SyjRGECO1a, SyGCaMP6f and H2B-GCaMP6f's constructs are shown in the Appendix.

All viruses were previously diluted on 1:100 in pre-warmed NBM and then 50µl of the final concentration was added in 2ml of NBM present in each well. Transductions were performed at DIV7. All the viruses, except SyjRCaMP1b, were transduced with a cell density of 130000 cells per well and MOI of 100. SyjRCaMP1b was transduced with a cell density of 60000 cell per well and an MOI of 200.
SyjRCaMP1b and SyjRGECO1a were transduced with a titre of $5.30x10^8$ using AAV-6-SyjRCaMP1b_WPRE (I) and AAV-6-SyjRGECO1a_WPRE (I) constructs, respectively. SyGCaMP6f was transduced using AAV-6-SyGCaMP6f (I) construct with titre of 6 $x10^8$ and H2B-GCaMP6f was transduced using AAV-6-H2B-GCaMP6f_WPRE (I) construct with titre of 4 $x10^8$.

Transfections

Actin-GCaMP6f's circular map and sequence are shown in Appendix

Transfections were performed in a 96 well-plate. The amount of DNA transfected was 1 µg for actin-GCaMP6f and 1µg for PSD95-mKate2 plasmid. It was transfected at DIV7.The protocol used was a calcium phosphate transient transfection protocol. 1µg of the DNA was added to a TE buffer solution and then 0.25M of $CaCl_2$ was mixed dropwise followed by a gentle vortex. This TE/DNA/$CaCl_2$ solution was added to a solution with the same volume of 2xHEPES buffered saline solution (HEBS), by pipetting in a dropwise mode, and then the

DNA-Calcium phosphate precipitate was incubated for 30 minutes protected from light, with 5-10 seconds vortex every 5 minutes, at room temperature. Half of the medium from the plate in use was removed and kept in the incubator separately for later (conditioned medium). After that, a 2 mM solution of Kynurenic acid (Sigma-Aldrich) in fresh medium was added to each well. Afterwards, the DNA-Calcium phosphate precipitate was added to each well in a dropwise mode and then the cells were incubated for one hour and half. Meanwhile it is important to check the cells in order to track the formation and distribution of the precipitates on the coverslips. Next, the medium within precipitates is removed and the cells were incubated with an acidic solution (2 mM Kynurenic acid and 3 mM HCl on fresh medium) for 17 minutes at 37 °C, in order to dissolve any precipitate left. Once dissolved, the precipitates were completely removed by washing the cells with fresh medium. The conditioned medium was then added to each well and it was incubated with half conditioned medium and half fresh one.
An immunocytochemistry was performed on the actin-GCaMp6f transfected neurons, in a 96-well plate and then it was imaged on Opera Phenix (See immunocytochemistry protocol).

Immunocytochemistry

Cells were fixed, under the hood, with 4%PFA/4%sucrose for ten minutes at room temperature and washed three times with 1xPBS. Then the permeabilization step was performed by adding 0.3% Tween20 and incubating the samples for ten minutes at room temperature. After that the cells were washed three times with 1xPBS, incubating for five minutes in each wash. Then it was added 1%BSA/0.3%Tween20 and the samples were incubated for 30 minutes protected from light in order to block unspecific binding of the antibodies. Afterwards, the primary antibodies were added to the cells at a dilution of 1:500 in 1%BSA/0.3%Tween20 for one hour at room temperature. Three times washings on 1xPBS were performed followed by the incubation of the secondary antibodies, which were diluted 1:500 in 1%BSA/0.3%Tween20, for one hour at room temperature and protected from light. Next, the cells were washed three times by incubating five minutes with 0.3% Tween20 each time, protected from light. Later, it was added Hoechst in a concentration of 10µg/ml per well before incubating the cells for 4 minutes. Dilutions of Hoechst (DAPI) were made on 0.3% Tween20. 1xPBs were used to rinse twice and the cells were keeping at 1xPBS until imaging analysis.
Primary antibodies used to H2B-GCaMP6f experiments were Anti-GFP AB5450 (goat) from ABCAM and Anti-NeuN ABN78 (rabbit) from Milipore. Additonally, secondary antibodies used were Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 and Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor® 488.
The immunostained 96 well-plate was scanned using the Opera Phenix™ high-throughput confocal microscope. The excitation was performed at 488nm, 561nm and 640 nm and 405nm for GFP, RFP, NeuN and DAPI imaging, respectively as well as use as emission filters respectively 500-550nm, 570-630nm, 650-760nm and 435-480nm.
To image basal fluorescence of **actin-GCaMP6f** experiments on Phenix, 12 planes with 0.5µm between planes were set up. The images were taken with binning 1 on 40X Water objective of 1.1NA and the exposure time to 488nm laser of 400 ms.

To image **H2B-GCaMP6f**, Anti-GFP antibody was excited by488nm laser, anti-NeuN antibody was excited by 561nm laser and DAPI was excited by 405nm laser. It was imaged 8 planes with 1µm between plans. The images were taken with binning 1 on 40X Water objective of 1.1NA and the exposure time to 488nm laser of 400ms.

Imaging synaptic activity in cultured hippocampal neurons

Hippocampal cultures were dissociated from E19 rat pups and transduced at DIV7 with the respective virus of interest. Experiments were performed between DIV14 and DIV30 in NES buffer at pH 7.4 containing (mM): 136 NaCl, 2.5 KCl, 10 HEPES, 1.3 MgCl2, 10 glucose, 2 CaCl2. To test the effect of synaptic blockers, 0.01 mM NBQX and 0.05mM D-AP5 (Tocris Bioscience) were added to the cultures.

Neurons were imaged in *vitro* using an EMCCD camera (Andor iXon ultra 897 High Speed EMCCD Camera) mounted on an Olympus IX 83 fluorescent microscope with 60x and 100x objectives with 1.25 NA and 1.30 NA oil immersion objective both, respectively. Images were captured in Frame Transfer mode at a depth of 16 bits using Andor SOLIS for Imaging: X-1086. In addition, for GCaMPs imaging the filter set used contained a FF01-474/27-25 excitation filter, FF495-Di03-25x36 dichroic mirror and a FF01-525/45-25 emission filter. For RCaMPs and RGECOs the filter set used contained FF01-562/40-25 excitation filter, FF495-Di03-25x36 dichroic mirror and FF01-624/40-25 emission filter. Illumination source used was LED (Omicron Laserage Laserprodukte GmbH, Rodgau, Germany) and it was regulated by Omicron Control Center (v3.5.30) software.

Trains of electrical stimulation pulses (APs; 1ms of duration, 10 mA of intensity) of different duration were applied through two parallel platinum wires 4 mm apart, which were built in a custom-built chamber and connected to an electrical stimulator. Camera, electrical stimulation, 477nm LED, and 555nm LED were all controlled by an open source pulse generator named PulsePal2[74] which was controlled using MATLAB 2013b. Trains of APs were all programmed to start with 2 seconds of delay relative to the beginning of the recording. For stimulation with different number of APs, all the recordings had 300 frames with 0.03 seconds of exposure time. Trains of 2, 3, 4, 5, 8, 10, 20 and 40 APs were delivered at a fixed frequency of 20Hz. In addition, to image responses to one single action potential a train of 5 pulses of 1AP 8 seconds apart of each one, called 1APseries, was delivered. Recordings were imported to Igor Pro 6.37 (Wavemetrics) and then analyzed.

Data analysis – Igor

Image sequence were imported to Igor Pro 6.37 (Wavemetric) in which the background signal was subtracted by choosing manually a displaced ROI where there was no stimulus-evoked fluorescence signal. Threshold was adjusted manually by Igor Correlation Map visualization and, from that threshold value, identification and management of ROIs was performed by Igor Segmentation. Signals were measured as $\Delta F/F_0$, considering F_0 the mean of the first 15 points of the wave. At this point an average wave is created from all the single waves of one condition (See figure 2.1). However, it is important to consider that there are waves that are interferences and artifacts that must be excluded. This exclusion used to be done manually

although that is not accurate and it could be considered bias by the user. To overcome this drawback, an Igor script was written to not only facilitate the data analysis of several recordings in terms of time saving and organization, but also to generate more accurate as well as not bias data analysis.
To get statistically significant biological results it is necessary more than one coverslip per culture, so instead of doing the analysis of all the recordings manually, the script can remove automatically waves which are two times the standard deviation away from the average as well as export directly from the average wave the parameters that are important to analyze and can also split the wave.

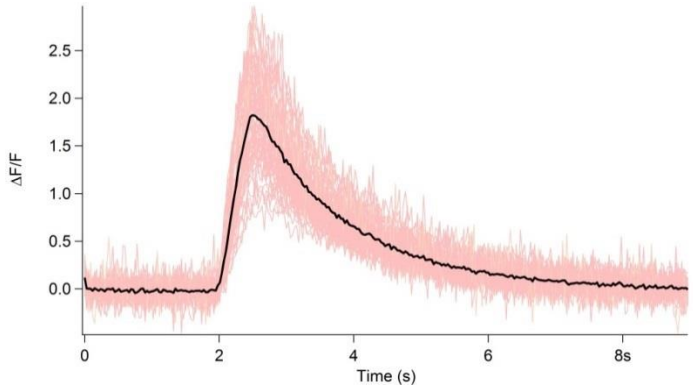


Figure 2.1- Example of an average wave from SyjRCaMP1b response to a train of 10 APs at 20 Hz in one coverslip. Example of an average wave (black trace) plotted over several individual 10 AP responses (red traces) from one coverslip using the script, so it is possible to have a visual confirmation by the user.

From a single average wave of each AP train the following parameters were measured:
Amplitude- the amplitude is the difference between basal fluorescence and the peak of fluorescence. To calculate amplitude values, the script will automatically pinpoint the maximum point of the wave and then subtract the average of the basal line right before the stimulus.
Linearity – this parameter is important to study the kinetics of the reporter and its saturation. After the exportation of the amplitude values to a text file, each average peak amplitude is plotted as a function of the trains of APs of different duration delivered.
Off kinetics (K_{off}) – Off kinetics is a constant of dissociation taken from the exponential decay of calcium from the reporter.
The script will pinpoint the maximum point of the average wave and run a quick fit Igor built-in function called *exp_XOffset* which is performed based on this decaying exponential equation:

$$y_0 + A\exp\left(\frac{x-x_0}{\tau}\right)$$

Example of the outcome of this function:

Coefficient values ± one standard deviation:		
	y0	=1.4282 ± 0.186
	A	=-1.115 ± 0.242
	tau	**=5.2746 ± 2.8**
Constant:		
	X0	=1

The fit coefficient tau is the decay constant of the Off-kinetics response.

Functional Analysis Panel

To easily perform all the possible tasks written on the script commands, a macro called Functional Analysis was created. It is a panel with several buttons, each one with a script's function associated. It is designed to start from the top and go down throughout all the possibilities. It starts for setting a folder with a name – **Set Folder** button. The buttons are designed for the analysis of recordings of trains of APs of various durations or even 1 AP series.

Regarding 1 AP series, each individual peak from 1AP series train is cropped and plotted together to make the average of the 5 peaks. To perform automatically the 1AP series analysis as well as the analysis of different trains of APs, the script creates several empty folders by clicking on **Create Folders** button. For extraction of all the $\Delta F/F_0$ waves (DF waves) from the raw data it is necessary to click on '**DF extraction'** button first. One of the empty folders is called "DF waves" and it is where all $\Delta F/F_0$ waves are placed; from this folder, after clicking on the '**split wave'** button, it creates another folder called **'DF extracted'** with each 1AP series wave splits into five different segments corresponding to the 1AP single peaks. The Split Wave button only works for 1APseries waves, although the rest of the buttons are for general use. From that extracted segments, the script automatically excludes waves with no peak that would be considered interferences, so another two folders are created: 'selected waves' and 'excluded waves' after you click on **DF selection** button. Now all the valid and not valid peaks from 1AP stimulus are stored separately for further analysis. The next step is to make the average wave of all the 1AP segments of 'selected waves' folder, by clicking '**Make Average'** button. After the click it automatically generates an average wave which is stored on a folder named '**Average waves'**.

Following the steps above for analysis of different trains of APs is also possible. The script will exclude waves taking into account the following parameters: 1. Waves that show amplitude two times higher and lower than the standard deviation and 2. waves with a baseline amplitude similar to the amplitude of the peak, so flat waves considered interferences can be excluded automatically (See script in Appendix). In the end an average wave is created, and can be plotted in a graph by clicking on **Plot Graphs** button.

To calculate the amplitude of each individual DF wave in one folder (i.e 10AP folder), the next buttons are focused on each individual DF wave of one single folder. There is a button called **'Calculate DF's Amplitude'** which will print a column with the value of each DF wave amplitude. By the time that an average wave is created, there are several functions (explained above) to use over the amplitude wave, such as **Amplitude**, **Area Under Curve**, **OffKinetics.** It is possible to print the results to a text file on the current folder of the computer, by clicking on **Print results** button**.** Afterwards, to not only save some memory but also to get several

folders (i.e 1AP series folder, 10 AP, 20 AP, 40 AP, etc) in the same experiment, two buttons named '**Delete Video**' and '**Kill All Graphs'** were designed to automatically delete the video and all the unnecessary graphs of the current folder so you can move forward the next one easily. To restart another video analysis, it is necessary to create another folder, so the button **Restore folder** will allow that on an easy and automatic way.
By the end, when all the folders (i.e 1AP folder, 2AP folder, 3 AP folder, etc..) have the respective average wave created and stored, the aim is to focus on the average wave of each condition. On the left side of the box there are buttons to apply the single wave functions to all the average waves created so far in the experiment. Those buttons are **Amplitude All**, **Plot All Averages**, **Off Kinetics All** and **Area All**. The script goes over all the different folders and run the functions above only on the average wave.
To guarantee the analysis of the same exact ROI throughout the videos, there is an option called **Copy AP10 ROI** which will take the ROIs from 10 AP video and copy to the current folder.

Figure 2.2- Functional Analysis Panel. Interface for the execution of the script functions during analysis of live imaging recordings. Each button is associated with a function previously programmed.

Pharmacology

Compounds

Three different compounds were used as modulators of neuronal excitability: KV1.3 channel blocker[9], LY450108[10] and a compound that was obtained from a phenotypic screening named Compound X due to confidentiality reasons.

All the compounds were dissolved in 100% DMSO on a final concentration of 10 mM, and stored at room temperature protected from light. For experiments, stocks were diluted first on the buffer used on the experiment (NES or NES++), as a pre-dilution stock, with the concentration three times the final concentration. The final concentrations used on the experiments were 10 µM, 3 µM or 1 µM, in 0.1% DMSO.

Imaging spontaneous activity

To test the effect of the compounds on the cultures, 120 seconds spontaneous activity recordings were performed before the addition of the compound, and then the same was performed at 2, 7, 15, 30, 45 and 60 minutes after the addition of the compound.

Compound addition was performed by taking out 100 µl of the 300 µl buffer where the cells are in and adding 100 µl of the pre-dilution of the compound. After that, time point's recordings are performed during one hour to follow the effect of the compound over time as described above. All the experiments of pharmacology were done on the same microscopy set up to avoid variability due to different equipment.

Spontaneous activity analysis

The spontaneous activity recordings have 3900 frames, exposure time of 0.03 s. The analysis of these videos was performed on Igor as well, with photo bleaching correction. Since the 120 s recordings were too large to load on Igor, some recordings were first divided on ImageJ to get two smaller movies with 2000 and 1900 frames to analyze on Igor software. On Igor, the background was subtracted and then, using the same ROI in all videos, raw waves were corrected for photobleaching and then concatenated on a final graph.

According to Royle SJ *et al.* (2008), the subtraction of a double exponential decaying function fitting the local background to the data traces is one way to correct for photobleaching[75].

3. Results

Genetically-encoded calcium indicators

SyjRCaMP1b

Characterization of SyjRCaMP1b

SyRCaMP1b is a genetically-encoded calcium indicator formed by a variant of a red fluorescent calcium reporter called RCaMP1b that is linked to a synaptophysin sequence. RCaMP1b is based on a thermostable red fluorescent protein named mRuby[29]. Fluorescent reporters linked to synaptophysin are commonly used to monitor pre-synaptic activity by tracking the influx of calcium through the synaptic channels[27].

To perform a full characterization of a reporter, several parameters need to be evaluated: 1. dynamic range, 2. sensitivity, 3 stability of responses over time, 4. kinetics and 5.expression of the reporter along the neuron.

SyjRCaMP1b expression

Knowing that mRuby fluorescence is excited at 558 nm[76], fluorescence of SyjRCaMP1b in rat cortical cultures plated into a 96-well plate was excited by a 561 nm laser on Opera Phenix. Even though SyjRCaMP1b belongs to the RFP-based reporter family which it is shown to have lower basal fluorescence than a GFP-reporter family[76] SyjRCaMP1b exhibited a visible basal fluorescence that allowed the study of its expression in cortical cultures (See Figure 7.1 in Appendix)

Dynamic range of SyjRCaMP1b

To analyze the dynamic range of responses of SyjRCaMP1b to electrical stimulation, trains with a progressively increased number of APs were delivered at 20 Hz, using electrical field stimulation (See *Imaging synaptic activity in cultured hippocampal neurons* in methods section). As shown in figure 3.1, the amplitude of the response to the electrical stimulus scales with the number of APs delivered.

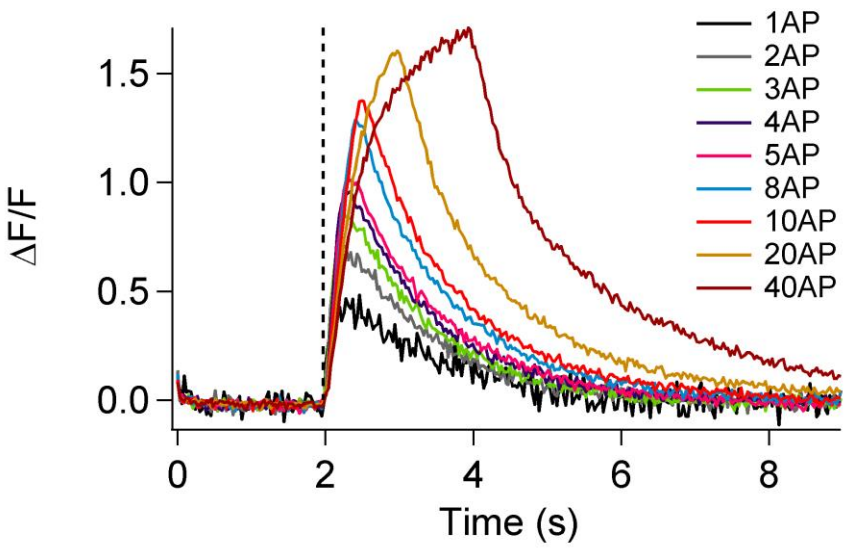


Figure 3.1- Dynamic range of SyjRCaMP1b in the absence of synaptic blockers. Average responses of SyjRCaMP1b to trains of 1, 2, 3, 4, 5,8, 10, 20, and 40 AP at 20 Hz. Each color trace is the average of 123 synapses from 3 different coverslips, at DIV14-16. Dashed line corresponds to the start time of the electrical stimulation.

Comparing linearity of SyjRCaMP1b to SyGCaMP6f and SyjRGECO1a.

Linearity is an important parameter for the characterization of a reporter because by knowing the responses' behavior it is possible to predict when the response is no longer linear and starts to saturate[19].

For optogenetic experiments, where channelrhodopsin-2 (ChR2) or its common variants are usually excited at 470 nm, to monitor synaptic calcium simultaneously, it is necessary to use a calcium reporter which is spectrally red-shifted. There are two such family of reporters: 1. Based on mApple (RGECO1a family)[77,78]; 2. Based on mRuby (RCaMP1b family)[24,29].

Even though RGECO1a was suggested to have higher affinity to calcium than RCaMP1b, RCaMP1b showed to be more addressable to perform optogenetics experiments combined with calcium imaging due to less photoactivation of RCaMP1b with blue light when compared to RGECO1a [23,24]. RCaMP1b's excitation range is spectrally more distant from ChR2 excitation spectrum than RGECO1 [23,24]. There are studies indicating that the use of RGECO1 combine with optogenetics can show artifacts as well as false positives due to its significant photoactivation with blue light [24,79].

It is important to compare SyjRCaMP1b with SyjRGECO1a because not only is linked to the same synaptophysin sequence but it also shows similar excitation and emission spectrum since they are both RFP-based pre-synaptic calcium reporters[23]. Nevertheless, SyjRCaMP1b and SyjRGECO1a structurally differ on the red fluorescent protein (RFP) they are based on– while SyjRGECO1a is based on mApple, SyjRCaMP1b uses mRuby as red fluorescent protein[23].

To study the linearity of the three different pre-synaptic reporters, the peak amplitude ($\Delta F/F_0$) of responses was plotted against the respective numbers of APs delivered[27].

To compare SyjRCaMP1b linearity with SyjRGECO1a, the responses of both reporters were plotted in graph 2A while the comparison between SyjRCaMP1b linearity and SyGCaMP6f linearity is shown is graph 2B.

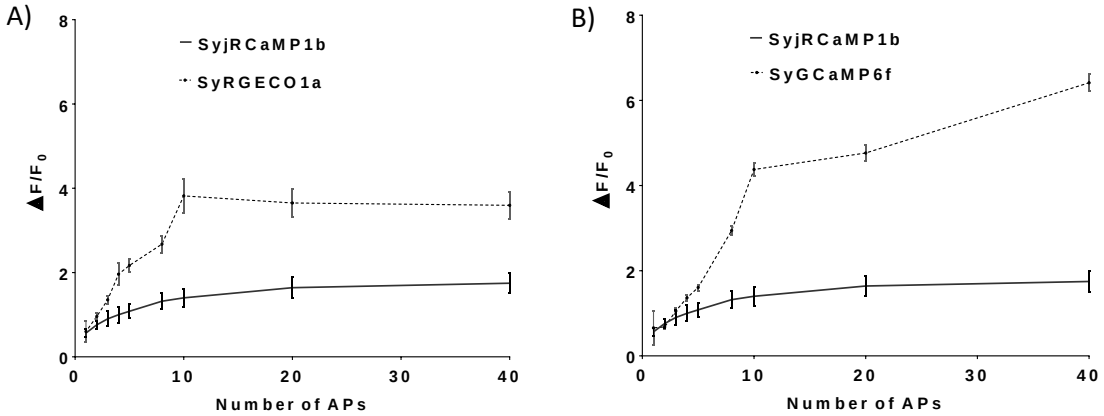


Figure 3.2- Comparison of pre-synaptic reporter's linearity (A) Peak amplitude of SyjRCaMP1b and SyjRGECO1a' responses as function of the number of APs delivered to each reporter. SyjRCaMP1b amplitudes were taken from graph 1 and shown as a continuous line while SyjRGECO1a average responses are shown as a dashed line which represents an average of 291 synapses from 4 different coverslips, two different regions per coverslip at DIV15. Error bars show s.e.m. (B) Peak amplitude of SyjRCaMP1b and SyGCaMP6f's responses as function of the number of APs delivered to each reporter. SyjRCaMP1b amplitudes were taken from graph 1 and it is shown as a continuous line while SyGCaMP6f's average responses are shown as a dashed line which represents an average of 177 synapses from 2 different coverslips at DIV16. Error bars show s.e.m.

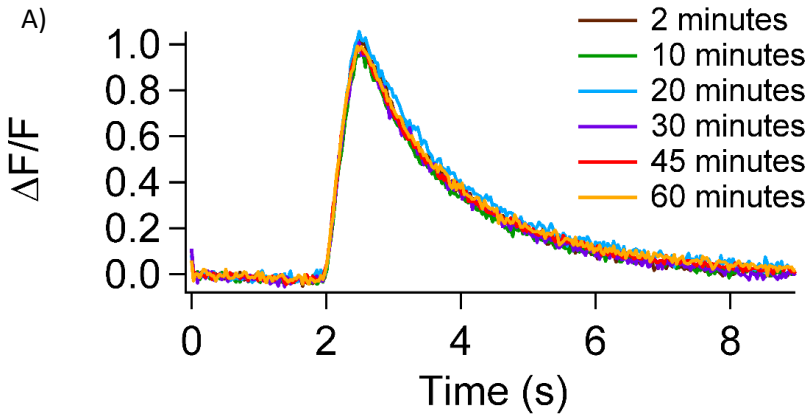
SyjRGECO1a show a saturation plateau from 10AP to 40AP at 20Hz while SyGCaMP6f shows an increase of ~20% from 10AP to 40AP.
Additionally, SyGCaMP6f and SyjRGECO1a have constantly higher peak of amplitudes than SyjRCaMP1b. Both SyjRGECO1a and SyGCaMP6f show an increase of ~40% to 10AP response while SyjRCaMP1b response to 10AP show an increase of ~10%.
Furthermore, SyGCaMP6f shows the highest peak amplitude to 40AP of the three pre-synaptic reporters.

SyjRCaMP1b's stability over time

Synaptic plasticity is not only short term synaptic modifications happening as a response of a chemical or electrical stimulus but it can also include longer lasting neuronal alterations over time[80]. To follow longer term alterations in synaptic activity, it is necessary to have a reporter showing stable responses to an electrical field stimulation over time. The common definition of long-term can be related to days and months yet performing live imaging without CO_2 perfusion system or temperature control, can lead to a stressful environment and consequently neuronal death in hours. Considering future possibilities of using this reporter in one hour synaptic function assays, it was delivered trains of 10APs at a frequency of 20 Hz at 2,10, 20, 30, 45 and 60 minutes, in the absence of synaptic blockers. The results show no

significant differences in amplitudes over time (figure 3.3) as well as overlapping in waves when plotted together (figure 3A).

A)



B)

Time (min)	ΔF/F
2	1.0625
10	0.99106
20	1.0582
30	1.0156
45	1.0074
60	1.0236

Figure 3.3- Stability of SyjRCaMP1b for one hour. A) Average of SyjRCaMP1b responses to trains of 10APs at a frequency of 20Hz after 2, 10, 20, 30, 45, 60 minutes, in NES buffer. Each trace represents an average of 61 synapses from one coverslip at DIV16. B) Average amplitudes' values taken from Figure 3.3A.

SyjRCaMP1b Kinetics

Off kinetics is a dissociation constant that represents the time of the decay of the calcium signal[81]. It can be measured in the average responses' graph by running an exponential decay function (more details in Methods) from the peak amplitude to the point where it reaches the basal line again. Off kinetics is an important parameter to characterize the shape of calcium transient[18].

Dissociation constants of SyjRCaMP1b to different trains of APs are shown in table A.

Table 3.1- Off Kinetics of SyjRCaMP1b. Table shows K_{off} correspondent to each peak amplitude from graph 3.1. Tau Coefficient, in seconds, are shown for each train of APs delivered. Tau coefficient is represented by "TauCoef" and is taken directly from the output of an Igor-built function associated to Off Kinetics (See Methods). The third column shows the standard deviation of the Tau coefficient shown as "TauStd".

K_{off}	TauCoef	TauStd
AP1	1.4388	0.093431
AP2	1.3276	0.02377
AP3	1.2735	0.016016
AP4	1.2463	0.012865
AP5	1.3226	0.011965
AP8	1.2313	0.008358
AP10	1.2465	0.008754
AP20	1.1752	0.01096
AP40	1.2492	0.023887

Actin-GCaMP6f

Knowing that one of the last neuronal transmission steps is accumulation of calcium in post synaptic spines, the study of post synaptic calcium activity is important to understand synapse on a functional and spatiotemporal level.
Alterations in neurotransmission can also happen in post synaptic spines. The well know LTP and LTD mechanisms are examples of activity-dependent plasticity on post synaptic spines[80]. To study post synaptic changes in calcium signaling, GECIs are commonly fused to post synaptic proteins as PSD-95 and Homer[82]. Although, Mao et al. have fused a GCaMP2 to a β-actin chicken sequence to image neural activity in post synaptic spines[33]. The authors mentioned drawbacks as the dimness of the signal when trying to localize spines[33]. To overcome this drawback, a more recent GCaMP – GCaMP6f – was fused with actin sequence to monitor calcium activity in post synaptic spines.

Actin-GCaMP6f Targeting

To be a valid post synaptic calcium reporter, actin-GCaMP6f must target post synaptic spines and be able to monitor changes in calcium influx. To evaluate actin-GCaMP6f targeting, a 96 well plate was co-transfected with Actin-GCaMMP6f and PSD-mKate2. mKate2 is a variant of red fluorescent protein(RFP)[76] which was linked to PSD to target only post synaptic spines, being considered a good and specific marker for post synaptic spines location. Using PSD-mKate2 co-transfected with Actin-GCaMP6fit is possible to study the expression as well as the targeting of this reporter.

It was used a 488 nm laser to image the expression of Actin-GCaMP6f (GFP-based reporter) and a 561 nm laser to image the expression of PSD-mKate2 (RFP-based probe), in Opera Phenix. Three different fields of the same well are shown below in figure 3.4.
I observed that PSD-mKate2 is expressed mostly in puncta while Actin-GCaMP6f is expressed along the neuron. The punctuated expression of PSD-mKate2 means it is targeting post synapses, while Actin-GCaMP6f is not well targeted to synapses, giving the idea that actin-GCaMP6f is expressed almost everywhere in the neuron.

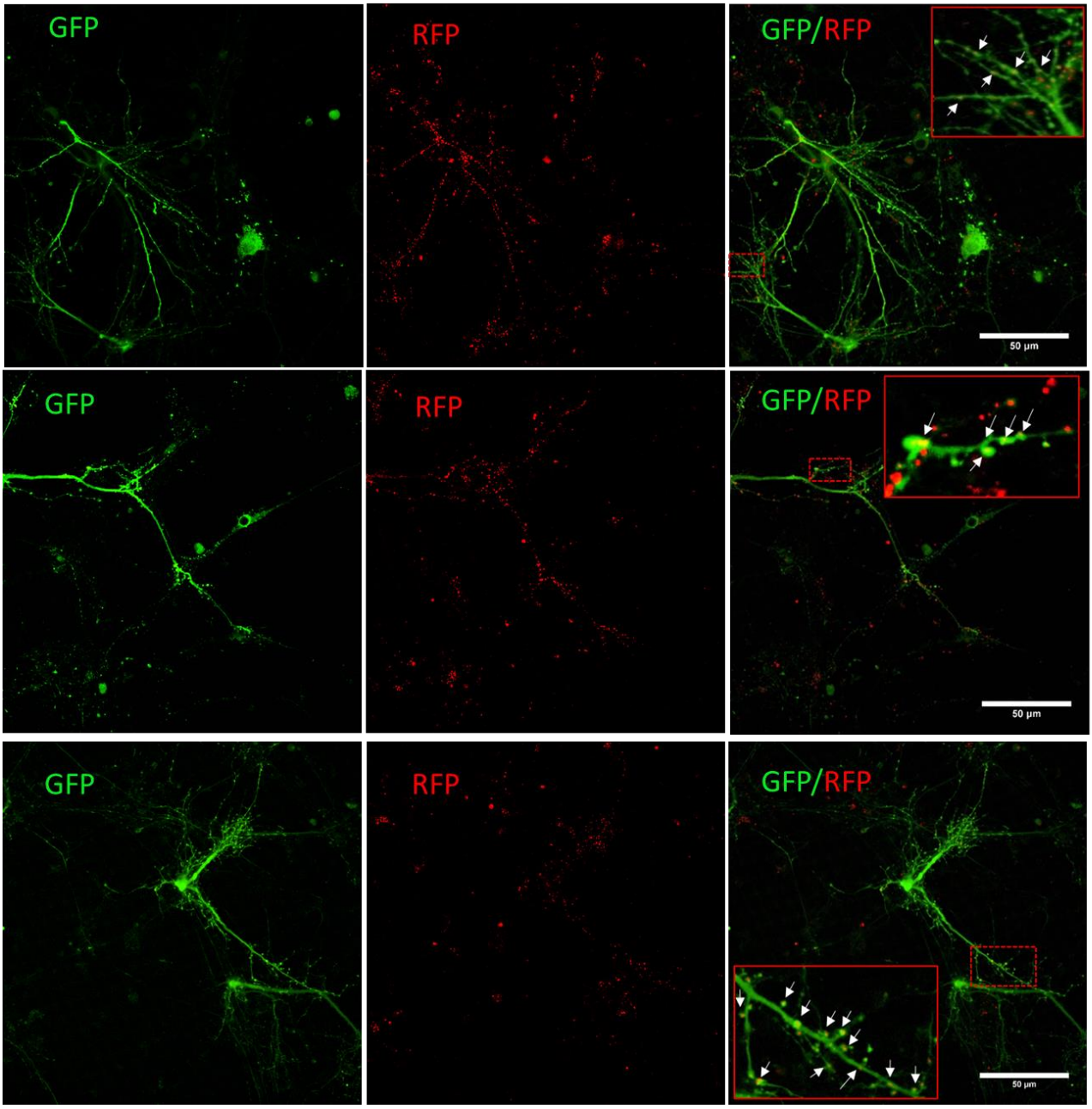


Figure 3.4- Basal Fluorescence of actin-GCaMP6f co-transfected with PSD-mKate2. Colocalization between Actin-GCaMP6f and PSD-mKate2 represented by white arrows.Transfections were performed on DIV7 using the calcium phosphate protocol (see Methods). Imaging was performed in DIV14. GFP from Actin-GCaMP6f was excited by 488nm laser and emission was collected using 500-550nm filter. For RFP, fluorescence was excited using 561 nm laser and emission was collected using 570-630nm filter. The last column shows the merge of the two previous images, showing the GFP from Actin-GCaMP6f and the RFP from PSD-mKate2. Each row represents a different field from the same well of a 96-well plate each. Images were taken with 40x water objective NA 1.1.

H2B-GCaMP6f

H2B-GCaMP6f is a green fluorescent reporter resulted from a fusion of GCaMP6f to H2B sequence, so it is a nuclear reporter [7]. The aim is not only to characterize H2B-GCaMP6f expression and dynamic range activity but also compare it to synaptic reporters as SyGCaMP6f and SyjRGECO1a. Nuclear responses are the integrated response of synaptic activity as well as a useful way to evaluate neuronal excitability of a network [7]. Neuronal excitability reflects the fundamental intrinsic properties of neurons and changes in intrinsic neuronal properties are shown to be related to CNS pathologies[4]- Regulation of neuronal transmission is not totally based on synaptic activity, intrinsic changes in membrane conductance of action potentials can also play a role in long term modulation of neuronal signals propagation. This nonsynaptic form of plasticity can influence the signal in the nucleus as well as dendrites and axons[80].

In addition, by studying changes in nuclear calcium signaling through addition of neuronal excitability modulators it is possible to pinpoint some intrinsic mechanisms of action of the compounds in neuronal excitability for further therapeutic uses[4].

H2B-GCaMP6f Expression

To study H2B-GCaMP6f expression on the neurons, basal fluorescence of H2B-GCaMP6f was tested first in detail (Figure 3.6). Secondly, an overview of eight fields was taken to evaluate the overall expression of H2B-GCaMP6f, with a MOI 100 (Figure 3.7).

H2B-GCaMP6f Basal fluorescence

From H2B-GCaMP6f basal fluorescence, it is possible to study the expression of H2B-GCaMP6f on hippocampal neurons since this reporter shows significant high values of basal fluorescent – from 400 to 2500 of fluorescence intensity.

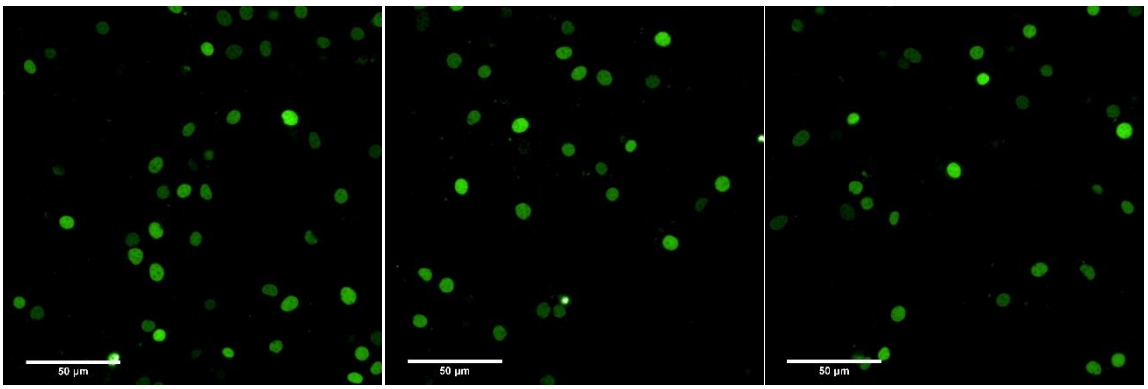


Figure 3.6- H2B-GCaMP6f Basal fluorescence. Three different fields in a given well of 96 well plate transduced with H2B-GCaMP6f at DIV7. H2B-GCaMP6f showed in green. Pictures were taken using Opera Phenix. with a 40x objective, and processed using Harmony 4.1 software. For GFP, fluorescence was excited using 488 nm laser and emission was collected using 500-550nm filter.

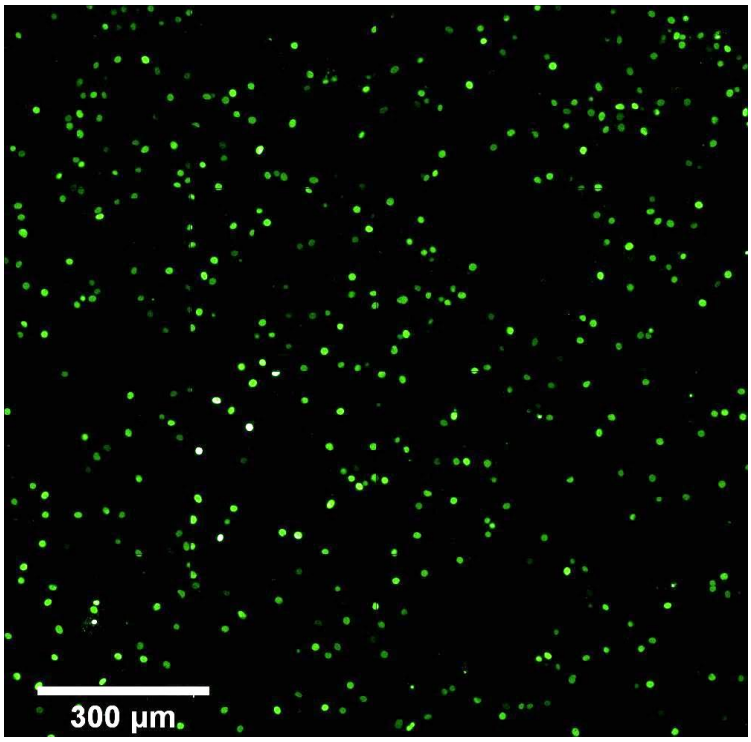


Figure 3.7. Overview H2B-GCaMP6f basal fluorescence. Basal fluorescence of the same well of 96 well plate used in figure 3.6. Basal fluorescence from H2B-GCaMP6f primary neuronal cultures in 96wp. GFP showed in green. Pictures were taken using Opera Phenix, with a 40x objective, and processed using Harmony 4.1 software. For GFP, fluorescence was excited using 488 nm laser and emission was collected using 500-550nm filter.

H2B-GCaMP6f Immunostainings

To study H2B-GCaMP6f nuclear expression and targeting, an immunocytochemistry was performed using a neuronal nuclear marker called NeuN. NeuN is a nuclear protein expressed specifically in vertebrate's neurons[83]. Antibodies against GFP and NeuN were used to check H2B-GCaMP6f and NeuN expression in two different biological replicates (Figure 3.8).
Counterstaining with DAPI helps to check the number of healthy cells on a given sample because DAPI binds double stranded DNA of every cells, including non-neuronal cells[84]. Neuronal viability of the culture was checked during experiments with counterstaining, although DAPI staining is only shown in the overview on Figure 3.9.

From the analysis of the merged pictures, every neuron labeled with H2B-GCaMP6f was co-localizing with nuclear marker NeuN, meaning that H2B-GCaMP6f is strictly expressed in neuron's nucleus.

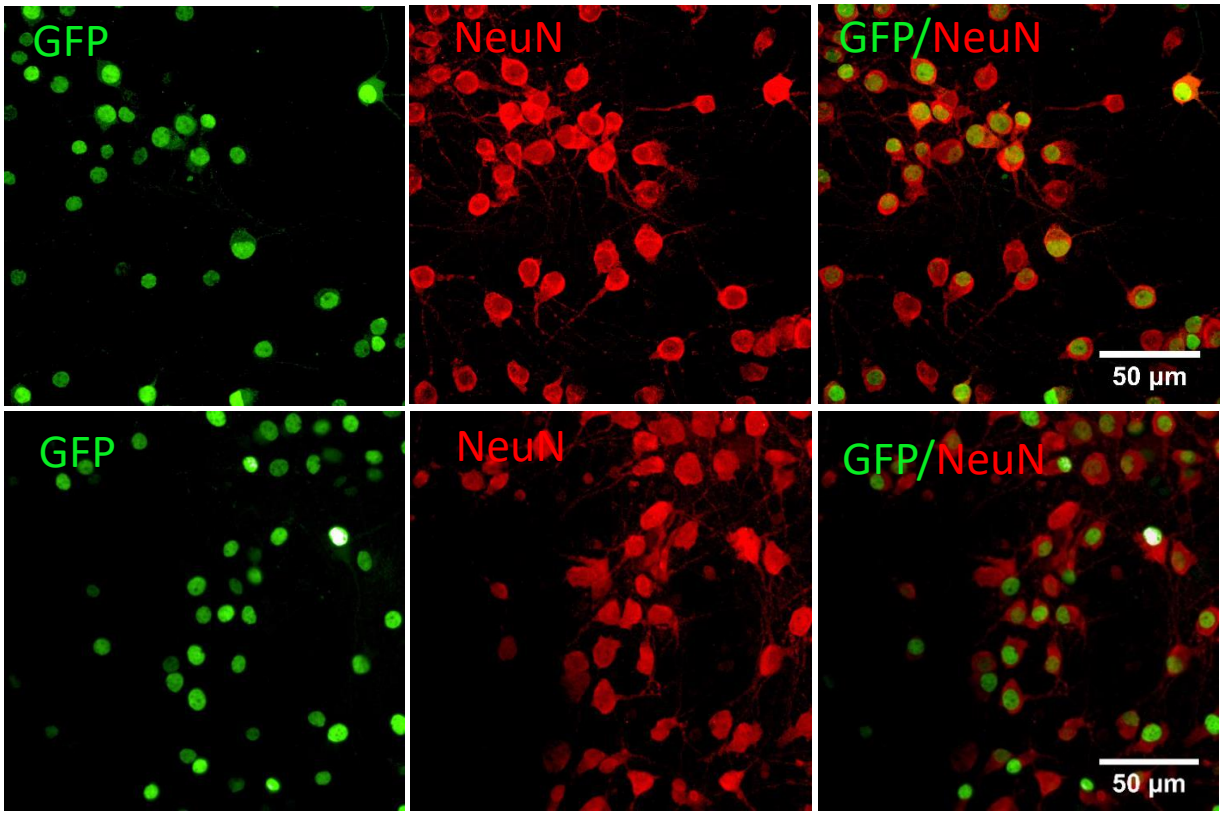


Figure 3.8- Immunostainings of H2B-GCaMP6f and NeuN in 96 well plate hippocampal primary neuronal cultures. Anti-GFP showed in green and anti-NeuN showed in red. Each row show a representative field of one well of a 96 well plate from a biological replicate. Last column show the merged pictures of each biological replicate. Pictures were taken using Opera Phenix, with a 40x water objective, and processed using Harmony 4.1 software. Fluorescence of H2B-GCaMP6f and NeuN were obtained by excitation at 488 nm and 561 nm laser and emission was collected using 500-550nm and 570-630nm filter, respectively. C is the merge of A and B showing overlap of nuclear labeling. Intensity shown with antibodies reach maximum of 10500.

To have an overview of H2B-GCaMP6f expression, eight fields of a well from a 96-well plate were imaged together (Figure 3.9). DAPI staining was also imaged so the cell viability of the culture was taken in consideration, however DAPI label not only neurons but also astrocytes[84]. Since cultures are heterogeneous, DAPI label neurons and non-neuronal cells while NeuN label only neurons' nucleus. In Figure 3.9, there is colocalization of H2B-GCaMP6f, NeuN and DAPI.

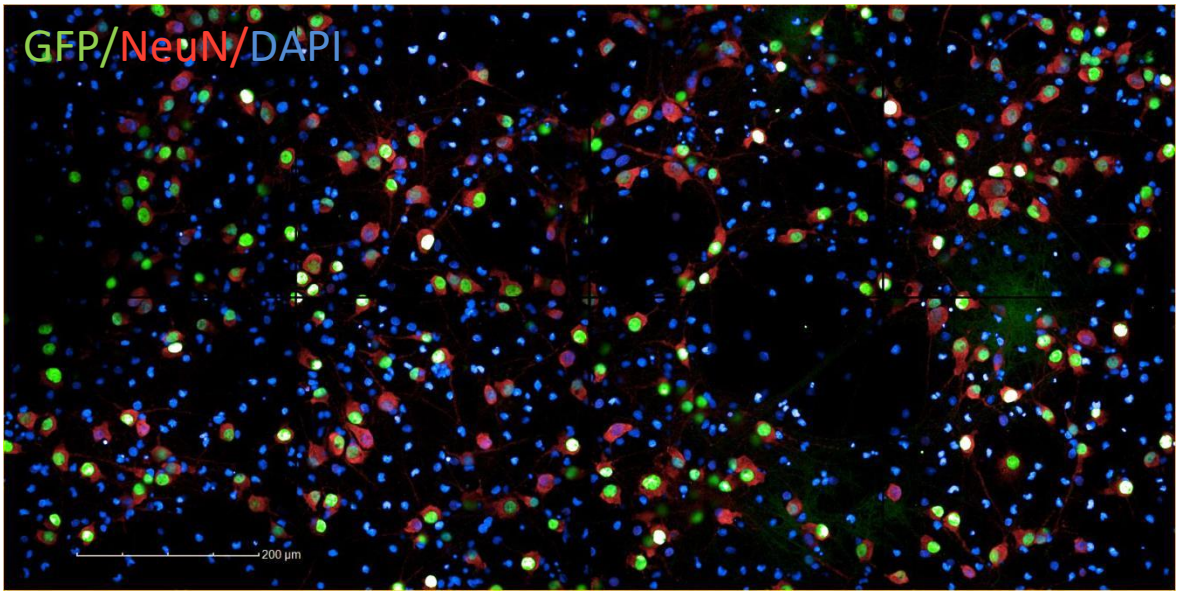


Figure 3.9- Overview of immunostainings of H2B-GCaMP6f, NeuN and DAPI from hippocamapal primary neuronal cultures in 96 well plate. Anti-GFP showed in green, anti-NeuN showed in red and DAPI in blue. Pictures were taken using Opera Phenix, with a 40x objective, and processed using Harmony 4.1 software. For H2B-GCaMP6f, fluorescence was excited using 488 nm laser and emission was collected using 500-550nm filter. For NeuN, fluorescence was excited using 561 nm laser and emission was collected using 570-630nm filter. For DAPI, fluorescence was excited using 405nm laser and emission was collected using 570-630nm filter.

Characterization of H2B-GCaMP6f

To characterize H2B-GCaMP6f the focus was the dynamic range and sensitivity in the presence and absence of synaptic blockers, and respective linearity's graphs. Every time it is referred the term "presence of synaptic blockers" it means a concentration of 0.01 mM NBQX and 0.05 mM D-AP5 which are antagonists of AMPA and NMDA receptors, respectively, in NES buffer – it can also be represented by NES++. In the absence of synaptic blockers, the condition is represented by NES.

H2B-GCaMP6f dynamic range in the presence of synaptic blockers.

To study the H2B-GCaMP6f dynamic range of activity, trains of APs of different duration were delivered at 20 Hz. In the presence of synaptic blockers, intrinsic neuronal responses are being studied because there is little basal network activity in the culture.

According to Figure 3.10, the peak amplitude of H2B-GCaMP6f responses progressively increases following the increase of APs trains' number, in the presence of synaptic blockers.

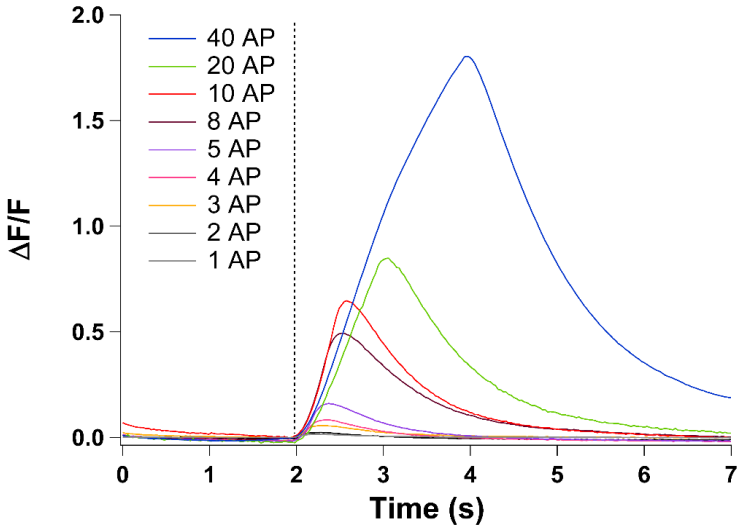


Figure 3.10- H2B-GCaMP6f dynamic range activity in the presence of synaptic blockers. Average responses of H2B-GCaMP6f to trains of 1, 2, 3, 4, 5,8, 10, 20, and 40 AP at 20 Hz. Each color trace is the average of 68 cell bodies from 4 different coverslips, two different regions per coverslip, at DIV16-20. Dashed line correspond to the start time of the electrical stimulation.

H2B-GCaMP6f dynamic range in the absence of synaptic blockers.

In the absence of synaptic blockers, the basal network activity is the normal network activity based on neuronal transmission meaning that calcium nuclear responses in this condition includes NMDA-evoked responses as well as AMPA-evoked ones 38.

It is visible in Figure 3.11 that in absence of synaptic blockers the signals do not show the same behavior as in the presence of synaptic blockers. For instance, the response to 40 APs train without synaptic blockers takes longer to reach basal line fluorescence values, comparing to the condition with synaptic blockers present in the buffer. Additionally, the response to 20 APs train shows a small peak in fluorescence late in time when it is getting close to reach the basal line. It is important to refer that the amplitudes in the absence of synaptic blockers does not scale with the number of APs delivered, as it does in the presence of synaptic blockers; for example, the peak amplitude to 10 AP train is smaller than the 8 AP's peak amplitude.

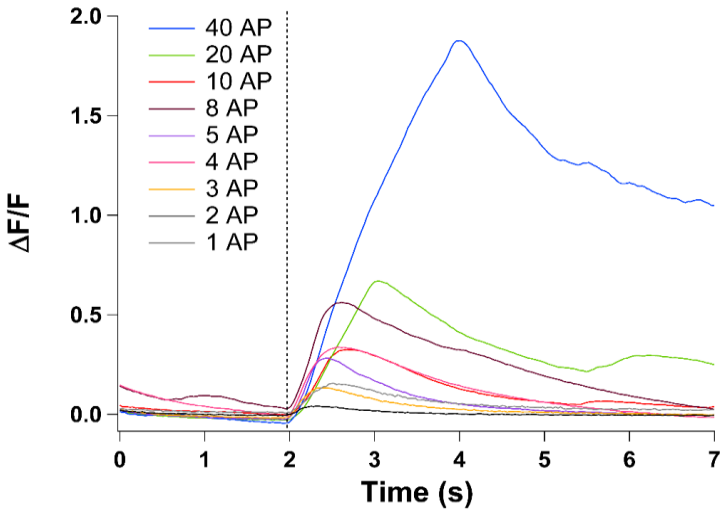


Figure 3.11- H2B-GCaMP6f dynamic range activity in the absence of synaptic blockers. Average responses of H2B-GCaMP6f to trains of 1, 2, 3, 4, 5,8, 10, 20, and 40 AP at 20 Hz. Each color trace is the average of 13 cell bodies from 2 different coverslips, two different regions per coverslip, at DIV21. Dashed line correspond to the start time of the electrical stimulation.

1 AP response in the presence and absence of synaptic blockers

A single action potential happens when membrane depolarization occurs due to the opening of voltage-dependent Na+ channels along the membrane, followed by hyperpolarization and then returning to resting potential. [85]. The phase after hyperpolarization is responsible for controlling excitability and may play an important role on the normal integration of neurotransmission since it is responsible for starting new APs or keep the firing rate continuous. The regulation of excitability and fire patterns is closely linked to K+ channels activity which play an important role in this phase[86]. The propagation of an action potential occurs across the membrane while induces local calcium signaling responses that will induce the opening of the perinuclear L-type_voltage gated calcium channels[50]. Changes in nuclear calcium signaling is possible to assay with a nuclear calcium reporter as H2B-GCaMP6f.

To study a single action potential, 5 trains of 1AP were delivered 8 s apart of each other and then each peak was isolated and plotted together to extract an average response for 1AP (See more details in Methods). This procedure was performed in the presence and absence of synaptic blockers to study the effect of synaptic inputs on a single action potential.
Waves used to plot the graph of 1AP response between presence and absence of synaptic blockers are the same from the experiments above - Figure 3.10 and Figure 3.11, respectively - which were isolated and plotted together in Figure 3.12.

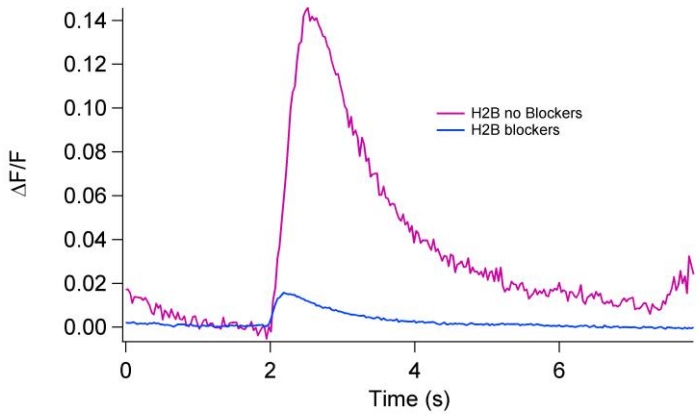


Figure 3.12- Average response of H2B-GCaMP6f to one single action potential in the presence and absence of synaptic blockers. Average responses to one single action potential in the presence ("H2B-GCaMP6FBlockers") and absence ("H2B-GCaMP6Fno Blockers") of synaptic blockers. In blue, the average peak amplitude of H2B-GCaMP6f in the absence of synaptic blockers as an average of 13 cell bodies responses from 2 different coverslips, two different regions per coverslip, at DIV21. In purple, it is shown the average peak amplitude of H2B-GCaMP6f in the presence of synaptic blockers as an average of 68 cell bodies from 6 different coverslips two different regions per coverslip, at DIV 16-20.

1AP response in the absence of synaptic blockers is ~12% higher than 1AP response in the presence of synaptic blockers. The fact that in the absence of synaptic blockers the neuron receives more than one input – synaptic and external electrical input - can explain the increase (See more details in Discussion)

Linearity

H2B-GCaMP6f in the presence and absence of synaptic blockers.

Taking into account the previous graphs – Figure 3.10 and 3.7- related to dynamic range of H2B-GCaMP6f, it was plotted the peak amplitude as a function of the number of APs delivered, in the presence and absence of synaptic blockers – conditions that can be referred as "Blockers" and "No Blockers" in the graphs, respectively. Figure 3.13 shows the differences in linearity between both conditions.

Regarding linearity, responses in the presence of synaptic blockers show a more linear pattern than the average responses in the absence of synaptic blockers. In the case of the absence of synaptic blockers the 1AP, 8AP and 10AP responses do not follow a linear pattern.

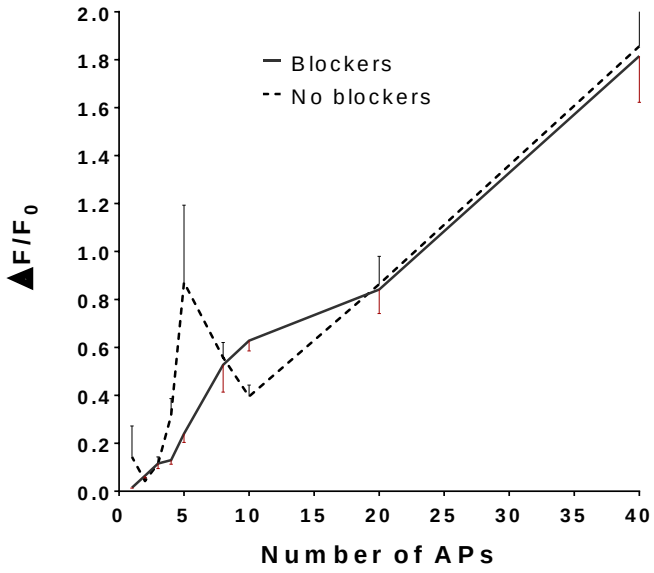


Figure 3.13- Comparison of H2B-GCaMP6f linearity in the presence and absence of synaptic blockers. Peak amplitudes of H2B-GCaMP6f responses in the absence of synaptic blockers as function of the number of APs delivered (dashed line). Peak amplitudes of H2B-GCaMP6f responses in the presence of synaptic blockers as function of the number of APs delivered (continuous line). Error bars show s.e.m.

From this graph, it is clear the difference between both linearities until 10AP responses. In the case of 1AP, while in the presence of synaptic blockers the response is small Taking 5AP average response as the most significant example, in the presence of synaptic blockers there is a peak amplitude showing an increase of ~20% while in the absence of synaptic blockers the amplitude reaches an increase of ~80% – four times more. In addition, from 10AP until 40 AP both conditions show the same linear pattern yet the values of amplitude from H2B-GCaMP6f in the absence of synaptic blockers are constantly higher than the values of H2B-GCaMP6f in the presence of synaptic blockers as well as persist longer, meaning that in the absence of synaptic blockers H2B-GCaMP6f responses take longer to reach the basal line.

Comparing H2B-GCaMP6f toSyGCaMP6f and SyjRGECO1a

It is important to compare dynamic range activity and sensitivity of different reporters. The choice of SyGCaMP6f for comparison to H2B-GCaMP6f is due to having the same calcium sensor – GCaMP6f22. Since SyGCaMP6f is a well-studied reporter and it only differs from H2B-GCaMP6f on the protein in which it is fused to, it makes SyGCaMP6f an interesting green spectral variant of a pre-synaptic calcium reporter to use as comparison to H2B-GCaMP6f.
The choice of SyjRGECO1a for comparison to H2B-GCaMP6f responses was due to be a red spectral variant pre-synaptic calcium reporter which consequently allow dual color imaging23. In addition, one of the aims is comparing nuclear responses with synaptic responses on the same region of neurons by using H2B-GCaMP6f as a nuclear calcium reporter from green spectral range and SyjRGECO1a as a pre-synaptic calcium reporter from red spectral range

(See Introduction for more details about excitation and emission in green and red spectra). It was first done a characterization of both comparison reporters – SyGCaMP6f and SyjRGECO1a.

Characterization of SyGCaMP6f and SyjRGECO1a

Trains of different number of APs were delivered at 20Hz to neuronal cultures previously transduced with SyGCaMP6f and SyjRGECO1a in which the last one was co-transduce with H2B-GCaMP6f to perform dual color imaging.

The focus was the condition with synaptic blockers, so all the experiments with SyGCaMP6f and SyjRGECO1a were performed in the presence of synaptic blockers - also referred as NES++.

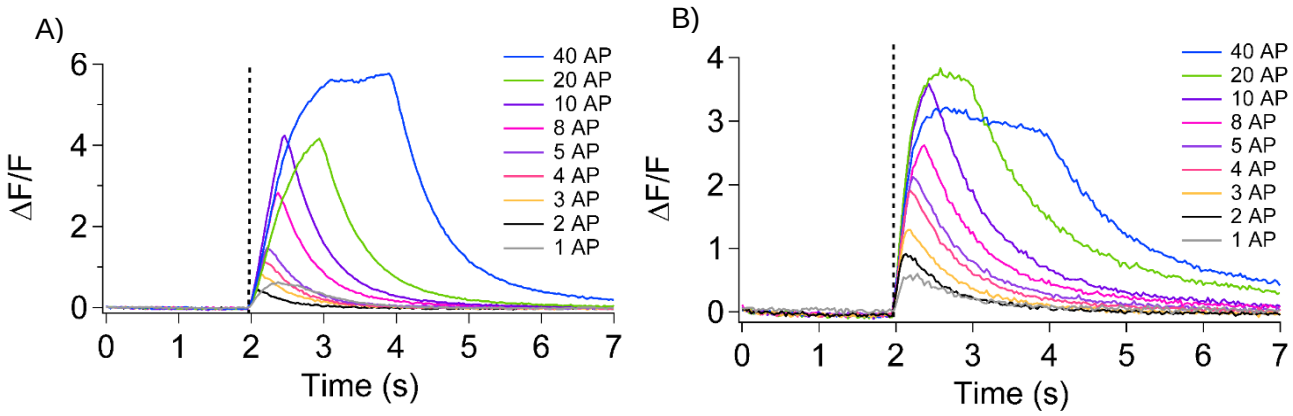


Figure 3.14-Dynamic range activity of SyGCaMP6f and SyjRGECO1a. (A) Average responses of SyGCaMP6f to trains of 1, 2, 3, 4, 5,8, 10, 20, and 40 AP at 20 Hz. Each color trace is the average of 177 synapses from 2 different coverslips at DIV21. Dashed lines correspond to the time of the electrical stimulation. Error bars show s.e.m. (B) Average responses of SyRGECO1a to trains of 1, 2, 3, 4, 5,8, 10, 20, and 40 AP at 20 Hz. Each color trace is the average of 291 synapses from 4 different coverslips, two different regions per coverslip at DIV15. Dashed line corresponds to the start time of the electrical stimulation.

In general, responses scale with the number of APs delivered in both SyGCaMP6f and SyjRGECO1a graphs (Figure 3.14). SyGCaMP6f shows not only a response to 1AP similar to the 2AP but also a response to 10AP with similar amplitude to 20AP. Interestingly, in SyjRGECO1a graph (Figure 3.14 B), 10AP response also show similar amplitude to 20AP yet it shows higher amplitude than 40AP.

SyGCaMP6f and SyjRGECO1a linearity

From dynamic range graphs, a linearity graph was made for both reporters, as shown in Figure 3.15.

SyjRGECO1a looks linear until 10AP and then it shows a saturation in responses from 10AP to 40AP responses (Figure 3.15), also confirming what was pointed out above on the dynamic range graph analysis (Figure 3.14 B) - the 40AP response is smaller than the 10 and 20AP ones.

According to the graph, SyGCaMP6f looks more linear and constant than SyjRGECO1a. In addition, SyGCaMP6f shows higher responses from 8AP to 40AP but not from 1AP to 8AP.

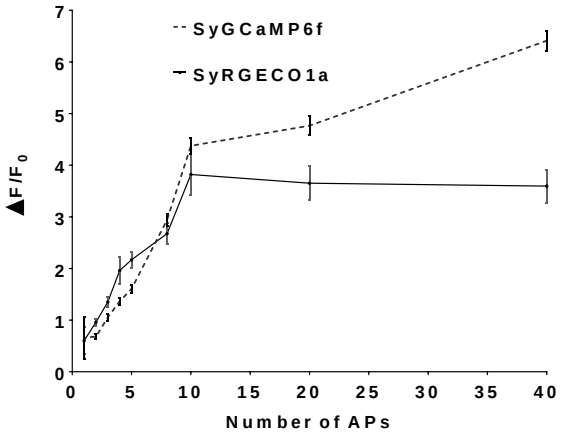


Figure 3.15- Comparison of SyGCaMP6f and SyjRGECO1a linearity. (A) Peak amplitude of SyGCaMP6 and SyjRGECO1a' responses as function of the number of APs delivered to each reporter. SyGCaMP6f's peak amplitudes were taken from Figure 3.14 A and shown as a dashed line while SyjRGECO1a average responses are taken from graph 10 B and shown as a continuous line. Error bars show s.e.m.

1AP responses of H2B-GCaMP6f, SyGCaMP6f and SyjRGECO1a

As explained before, it is important to compare the shape of one single action potential when characterizing different reporters. So far these three reporters - SyGCaMP6f, SyjRGECO1a and H2B-GCaMP6f - in NES++ and NES- were characterized. We plotted 1AP response from all the reporters characterized so far to evaluate the differences between green and red spectral variant of presynaptic reporter's responses - SyGCaMP6f and SyjRGECO1a- as well as differences on synaptic and nuclear responses -SyGCaMP6f and H2B-GCaMP6f. In addition, we also compared responses from H2B-GCaMP6 in the absence of blockers to the three different reporters responses in the presence of blockers. (Figure 3.16).

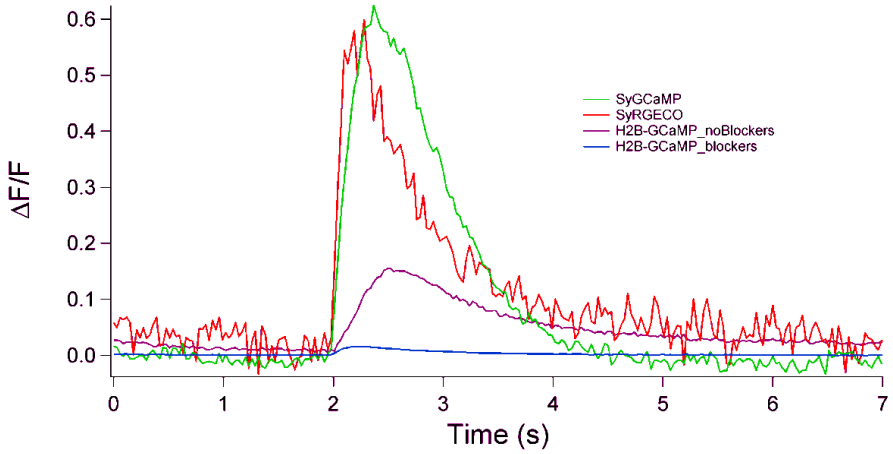


Figure 3.16- Average response of H2B-GCaMP6f, SyGCaMP6f and SyjRGECO1a to one single action potential. Blue trace represents the average peak amplitude of H2B-GCaMP6f to one single action potential in the presence of synaptic blockers ("H2B-GCaMP_blockers") and the purple trace represents the H2B-GCaMP6f peak amplitude but in the absence of synaptic blockers ("H2B-GCaMP6f_noBlockers"). Green trace shows the average peak amplitude of SyGCaMP6f in the presence of synaptic blockers and the red trace shows the average peak amplitude of SyjRGECO1a in the presence of synaptic blockers.

For pre-synaptic reporters responses– SyGCaMP6f and SyjRGECO1a - the 1AP responses are significantly larger than the 1 AP nuclear response of H2B-GCaMP6f. The difference between 1AP amplitudes of SyGCaMP6f and SyjRGECO1a is not as different as comparing nuclear and synaptic responses, yet SyGCaMP6f response to one single action potential is slightly broader than SyjRGECO1a.

Comparison of nuclear and synaptic signals

To focus on the differences between synaptic responses and nuclear responses multiplex imaging was performed. For that, SyjRGECO1a and H2B-GCaMP6f were co-transduced to get both reporter as close as possible of being in the same neuron, however there is no guarantees that the same neuron was co-transduced with synaptic and nuclear reporters. On live imaging, there was a visual confirmation that at least H2B-GCaMP6f and SyjRGECO1a were not overlapping; H2B-GCaMP6f was strictly expressed on the nucleus while SyjRGECO1a was targeting synapses (figure 3.17). On multiplex imaging, H2B-GCaMP6f was excited with 470nm LED and SyjRGECO1a was excited at 555nm LED. Three different regions of one coverslip transduced only with H2B-GCaMP6f was excited at 555nm LED and stimulated with a train of 10AP at 20Hz, to confirm that there was no photoactivation of H2B-GCaMP6f with the SyjRGECO1a excitation wavelength. There was no significant interferences (data shown in *Red interferences of H2B-GCaMP6f* in Appendix) so H2B-GCaMP6f responses were not influenced by SyjRGECO1a excitation.

Trains of different number of APs were delivered at 20 Hz on the H2B-GCaMP6f and SyjRGECO1a co-transduced cultures and the focus was the 1 and 10 AP responses in NES++. Since 10AP response is more visible in recordings, frames of 10 AP responses are shown below in four different time points. From 250 frames recording, the frames shown in Figure 3.17 are frames 67, 70, 90 and 250.

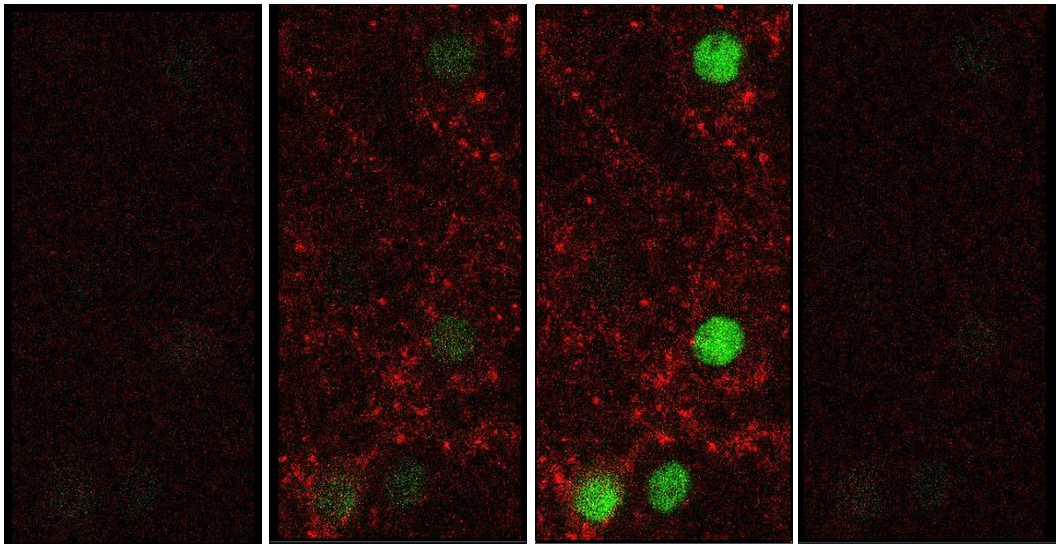


Figure 3.17 –Sequential frames of 10AP response of H2B-GCaMP6f and SyjRGECO1a in dual color calcium imaging. First image represents one frame of the region of interest to perform dual color live imaging before the delivery of one train of 10 APs at the frame number 67. The second picture is from frame number 70, approximately 0.09s after the 10AP train was delivered, showing the synaptic neuronal response in which the red puncta are transduced with SyjRGECO1a and the green areas are transduced with H2B-GCaMP6f. The third picture is taken from frame number 90, approximately 0.6s after the 10 AP train delivery and 20 frames after the second picture, showing the delay of nuclear responses comparing to synaptic responses. The last image is taken from the last frame of the recoding, almost 5 seconds after the 10 AP train stimulation.

10AP nuclear and synaptic responses

To a detailed comparison of nuclear and synaptic responses, 10AP's peak amplitudes were isolated from SyjRGECO1a and H2B-GCaMP6f's dynamic range graphs (Figure 3.14 B and Figure 3.10, respectively) and plotted in two separated graphs. Figure 3.18 shows the comparison between nuclear and pre-synaptic response to a train of 10APs.

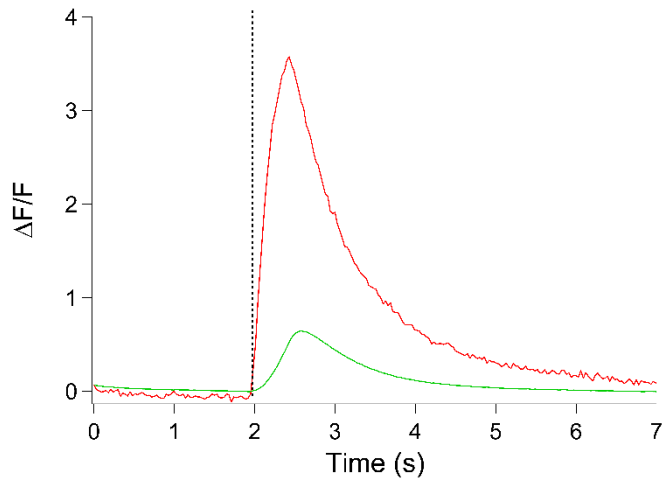


Figure 3.18- Comparison between nuclear and pre-synaptic responses to 10 AP. Average responses of H2B-GCaMP6f (green) and SyjRGECO1a (red) to a train of 10 APs at 20Hz. Dashed line corresponds to the start time of the electrical stimulation.

Linearity of nuclear and synaptic reporters

To compare the linearity between pre-synaptic reporters and nuclear reporters, it was plotted the peak amplitude of H2B-GCaMP6f and SyjRGECO1a responses as a function of the trains with different number of AP, in one single graph (Figure 3.19). It is visible in Figure 3.19 that the responses of the pre-synaptic reporter - SyjRGECO1a - are constantly higher than the responses of nuclear re porter - H2B-GCaMP6f. On the other hand, SyjRGECO1a shows a saturation plateau from 10AP to 40AP while H2B-GCaMP6f do not show any saturation plateau, on the contrary it shows a linear response from 10AP to 40AP.

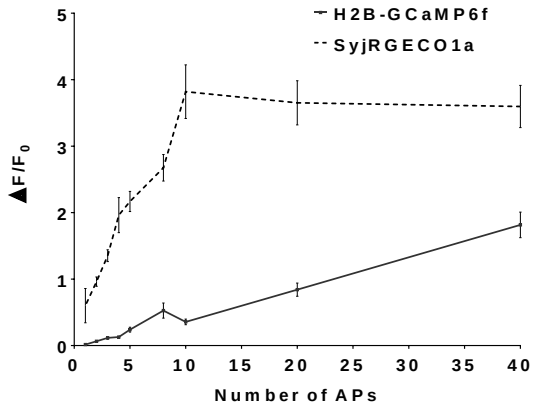


Figure 3.19- Comparison of H2B-GCaMP6f and SyjRGECO1a linearity. (A) Peak amplitude of H2BGCaMP6 and SyjRGECO1a' responses as function of the number of APs delivered to each reporter. H2B-GCaMP6f's peak amplitudes were taken from Figure 3.10 and shown as a continuous line while SyjRGECO1a average responses are taken from Figure 3.14 B and shown as a dashed line. Error bars show s.e.m.

Comparison of H2B-GCaMP6f and SyGCaMP6f

Since H2B-GCaMP6f and SyGCaMP6f are both spectrally green variants of GECIs it is not recommend to do co-transductions and dual color imaging as it was done with the SyjRGECO1a. H2B-GCaMP6f and SyGCaMP6f were individually compared, by plotting their linearities on Figure 3.20. SyGCaMP6f responses are constantly higher than H2B-GCaMP6f responses, yet both reporters exhibit a linear response.

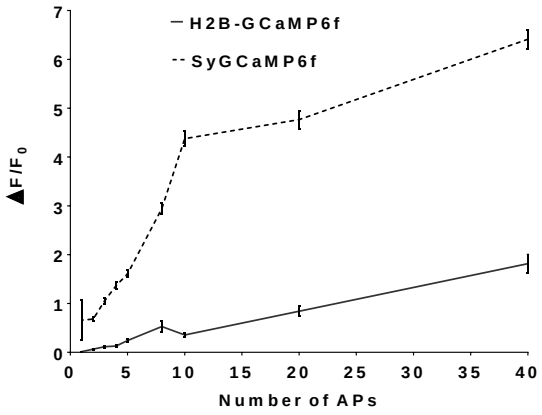


Figure 3.20- Comparison of H2B-GCaMP6f and SyGCaMP6f linearity. (A) Peak amplitude of H2BGCaMP6 and SyGCaMP6f' responses as function of the number of APs delivered to each reporter. H2B-GCaMP6f's peak amplitudes were taken from Figure 3.10 and shown as a continuous line while SyGCaMP6f's average responses are taken from graph 10 A and shown as a dashed line. Error bars show s.e.m.

Pharmacological characterization of H2B-GCaMP6f

To further characterize H2B-GCaMP6f, modulators of synaptic function were used in pharmacologic assays.

The focus was the study of neuronal excitability using three different compounds – LY450108, KV1.3 blocker and Compound X. One is an AMPA positive modulator - LY450108[10], while the KV1.3 blocker is an antagonist of voltage dependent potassium channel[9]. Both compounds are explained in detail below. No information can be given about Compound X due to confidentiality issues.

Taking into account that H2B-GCaMP6 and SyGCaMP6f were characterized before, the aim is using H2B-GCaMP6f as nuclear reporter to analyze neuronal excitability and to use SyGCaMP6f to compare the effect of the compound on synapses. Neuronal excitability is the combination of synaptic plasticity with intrinsic membrane properties [4], to study the two parts separately two conditions were created - experiments in the absence of synaptic blockers (NES) and the same experiment in the presence of synaptic blockers (NES++).

Knowing that synaptic inputs are necessary to generate an action potential[38], in the presence of synaptic blockers, NMDA and AMPA receptors are blocked meaning that there are no synaptic inputs to induce an action potential so the network activity shown is basal. However compounds can alter intrinsic membrane properties which will result in changes in the firing threshold or the firing pattern[4]. In conclusion, with synaptic blockers what it is being measured is neuronal intrinsic membrane properties.

In the absence of synaptic blockers, NMDA and AMPA receptors are actively generating action potentials so it is possible to visualize spontaneous activity and measure it with H2B-GCaMP6f as well as with SyGCaMP6f. Resuming, in the absence of synaptic blockers what is being measure is synaptic activity and plasticity over time.

With H2B-GCaMP6f as a nuclear calcium reporter the calcium influx measured at the nucleus is the integrated calcium response from synaptic NMDA receptors and AMPA receptors activation as well the activation of voltage-dependent calcium channels induced by membrane depolarization[49,50].
While the experiments were running, it was noticeable that cell bodies transduced with H2B-GCaMP6f placed side by side showed variability between them as a response to the compound in the same conditions. In addition, it was possible to visualize synapses from the same region reacting with different patterns to the same compound, in the same conditions.

Variability is an important factor that is not taken in consideration sometimes, so the aim is also show the variability not only between cell bodies or synapses from the same coverslips but also between coverslips, and finally show what it is missed if just the average of all coverslips is shown as a final result. To do that, it was also isolated some representative examples of different individual pattern of responses from one representative coverslip.

Experiment Design

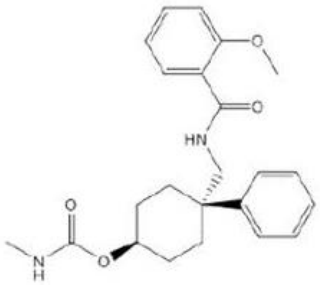
To perform pharmacology studies focused on neuronal excitability, spontaneous activity was recorded for 120 seconds over a time course. First of all, a train of 10 APs at 20Hz – named as" 10AP" - was delivery to check reactivity of the neurons. Secondly, live imaging recordings were taken before the addition of the compound and named as "Baseline" next to the graphs. Then the compound was added and time points at 2, 7, 15, 30, 45 and 60 minutes were recorded after the addition of the compound to the culture and labeled as "2 minutes", "7 minutes", "15 minutes", "30 minutes", "45 minutes" and "60 minutes", respectively. The title of each experiment is shown as: reporter name, compound name, compound concentration, condition of the experiment (NES or NES++) and the type of graphs shown (Average or Representative Examples).

Several time points were recorded not only in different conditions – NES or NES ++ - but also with different concentration of the compounds -10µM,3µM and 1µM and different reporters. All the 120 seconds' recordings have 3900 frames at 0.03 seconds of time exposure each.

KV1.3 blocker

KV1.3 is a voltage gated potassium channel that is involved in human T-cells resting potential [9]. K1.3 channels has been a therapeutic target mainly for immunosuppression strategies[87] as well as it has been involved as a target for multiple sclerosis treatments[88] It was shown that the blockade of KV1.3 channel, by benzamine derivatives, leads to membrane depolarization[9]. There is a benzamine derivative used as a K1.3 channel blocker in Miao et al. (2003) which was shown to be an enhancer of post synaptic calcium influx, assessed in previous PSD-GCaMP6f assays[9].

It was used a commercially available analogue to analyze neuronal activity. The chemical structure of the K1.3 blocker analogue is in Figure 3.21.



From Miao et al. (2003) Bioorg Med Chem Lett.13(6):1161-4 (cmpd #18)

Figure 3.21- KV1.3 blocker's chemical structure. KV1.3 blocker used is a (1r,4r)-4-((2-methoxybenzamido)methyl)-4-phenylcyclohexyl methylcarbamate, an analogue of the compound of Miao et al. (2003) publication.

In **Figure 3.22**, from 3 coverslips just one coverslip shows a significant response to 10 µM of KV1.3 blocker. It shows no spontaneous activity before the compound and then two minutes after neurons started to fire.

In **Figure 3.23**, it is noteworthy that from the reacting coverslip (CS1) just one cell body shows the pattern in the average. It is not possible to tell by the average that two coverslips did not respond and the reactive example was an isolated case from 11 cell bodies.

Since experiments were performed in the presence of synaptic blockers NES++) the focus were assaying the intrinsic changes on membrane properties, and there is one cell body (Cell body C) from 11 that show significant changes in spontaneous activity over time with the addition of 10 µM KV1.3 blocker.

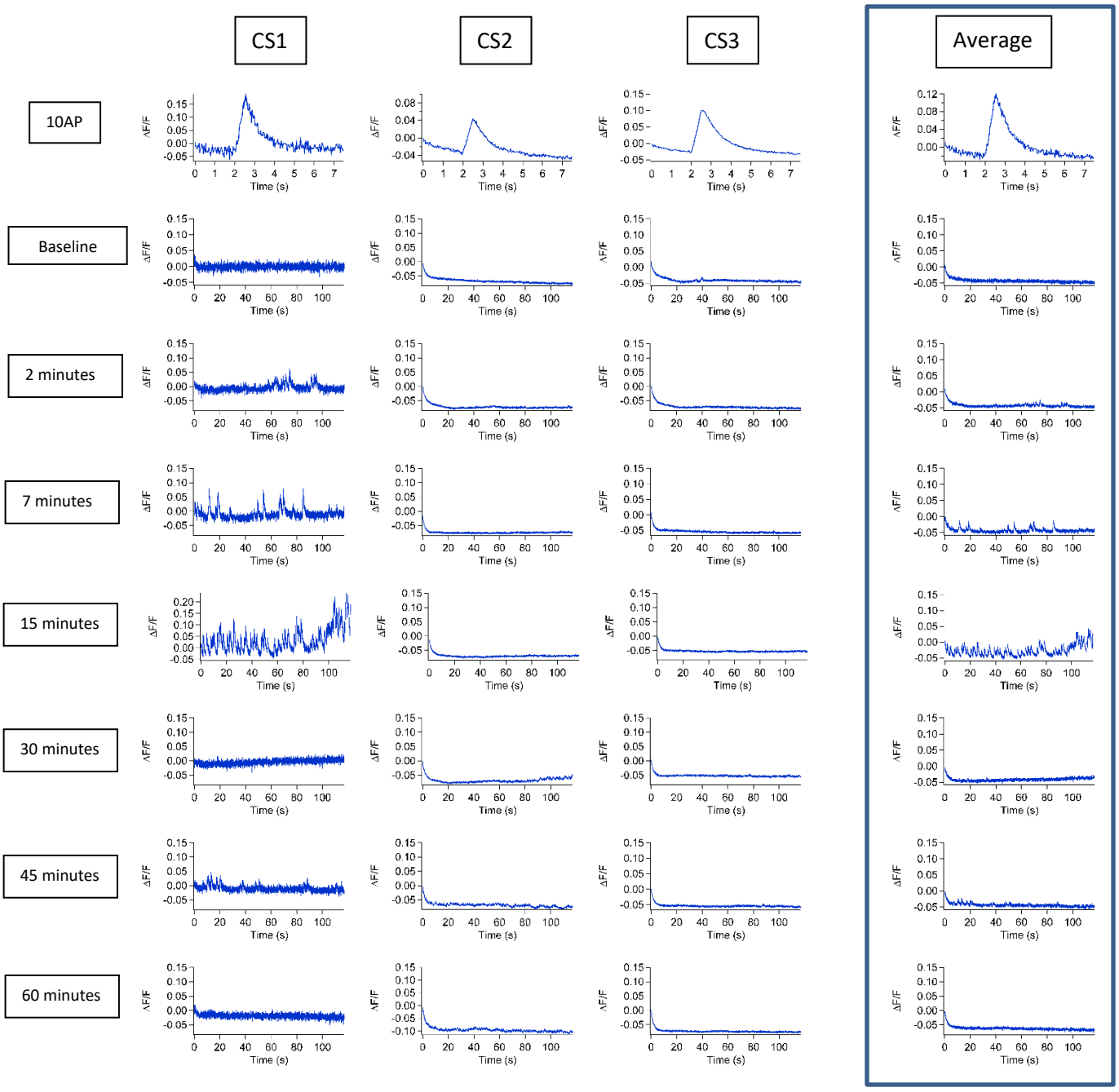


Figure 3.22- Overview of the effect of 10µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 3 reacting cell bodies at DIV 23, CS2 showed 4 reacting cell bodies at DIV23 and CS3 had 4 reacting cell bodies at DIV23. The total of cell bodies on this condition is 11.

H2B-GCaMP6f KV1.3 blocker10µM NES++ **Representative examples CS1**

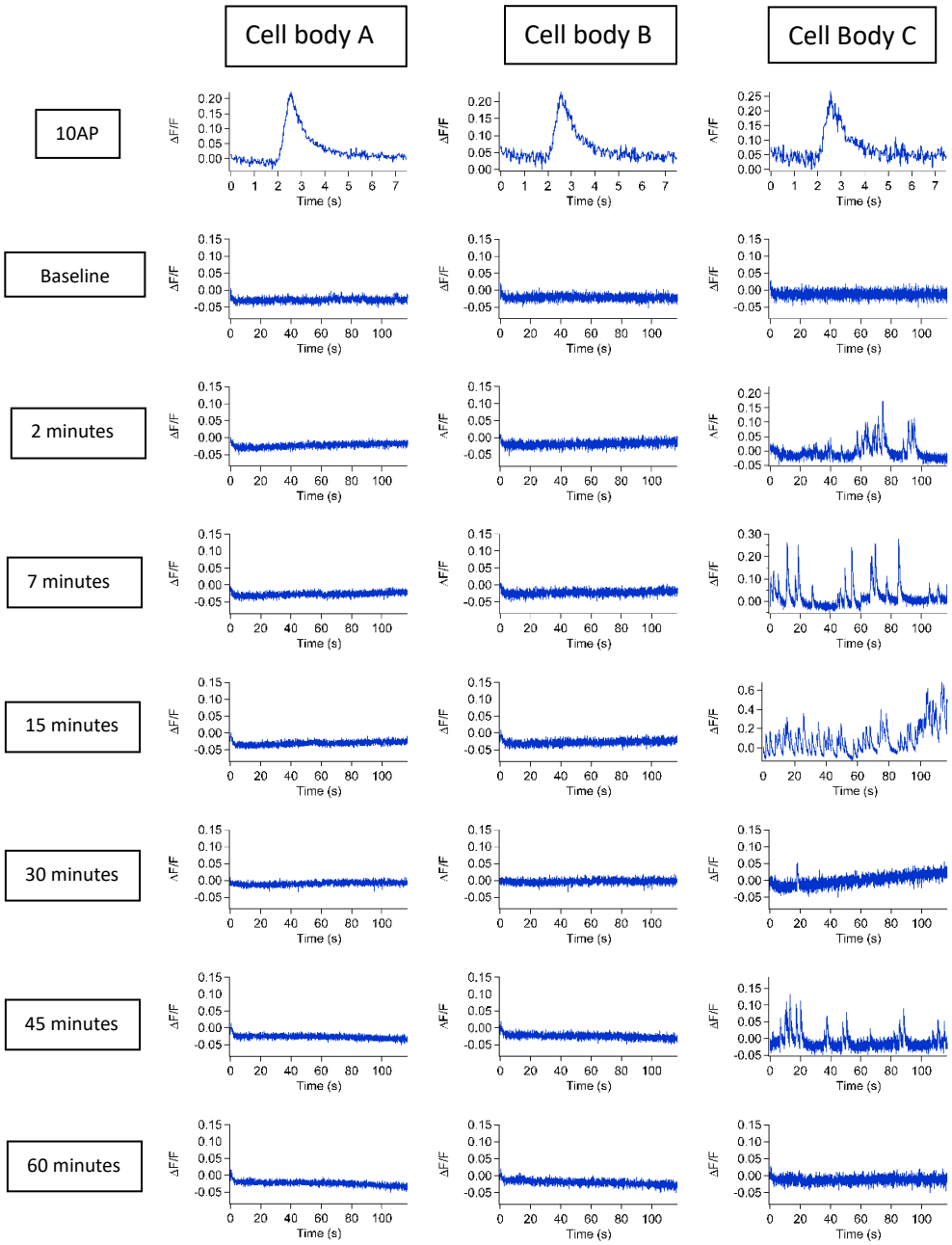


Figure 3.23- Overview of individual effect of 10µM of KV1.3 blocker compound on H2B-GCaMP6f. Representative examples of individual responses of three different side by side cell bodies label as “Cell Body A”, “Cell Body B” and “Cell body C” on the figure) of CS1, in the presence of synaptic blockers. Each column corresponds to an individual cell body’s response over time.

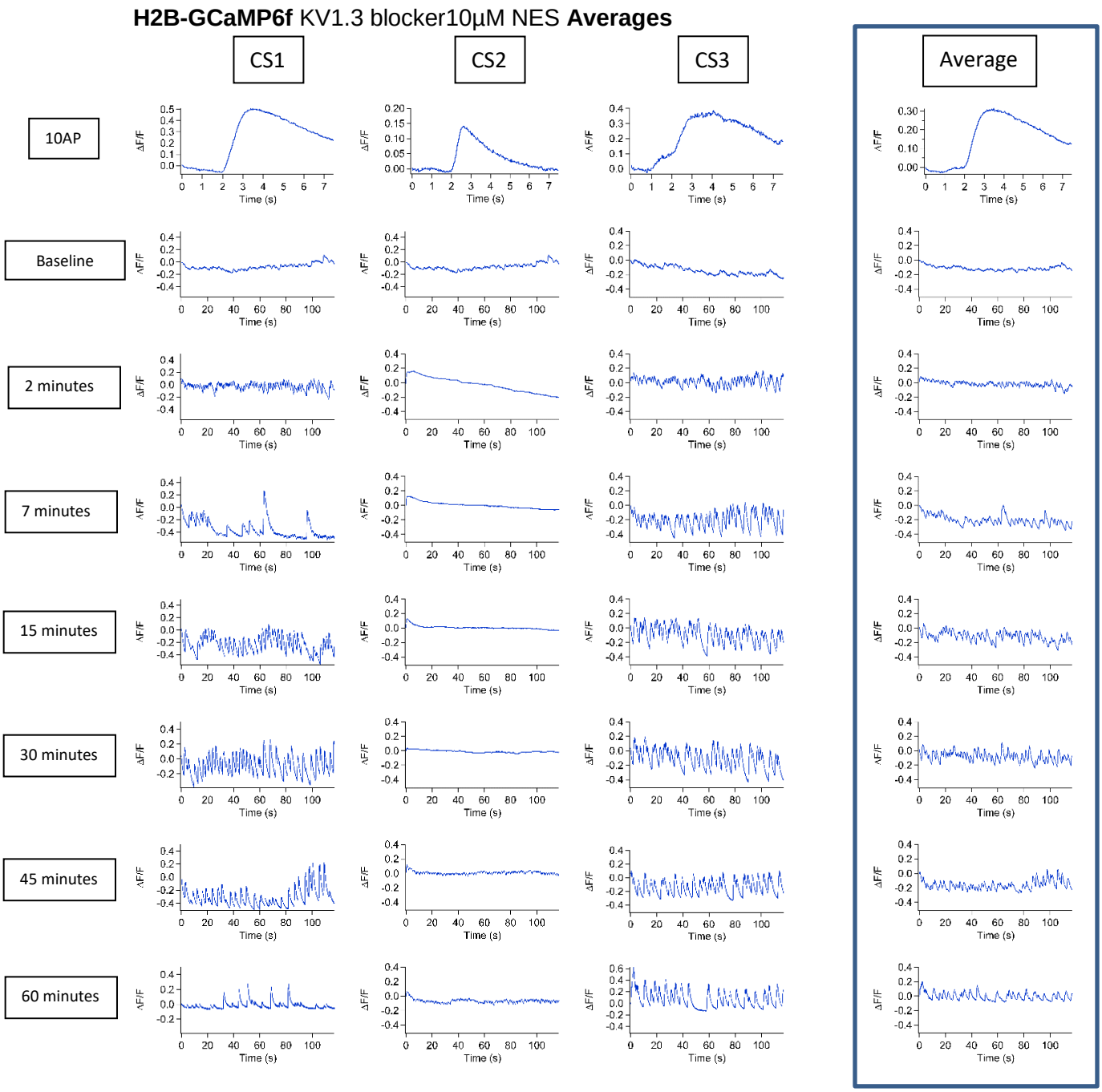


Figure 3.24- Overview of the effect of 10µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 2 reacting cell bodies at DIV17, CS2 showed 3 reacting cell bodies at DIV28 and CS3 has 1 reacting cell body at DIV15. The total of cell bodies on this condition is 6.

H2B-GCaMP6f KV1.3 blocker10µM NES **Representative Examples CS1**

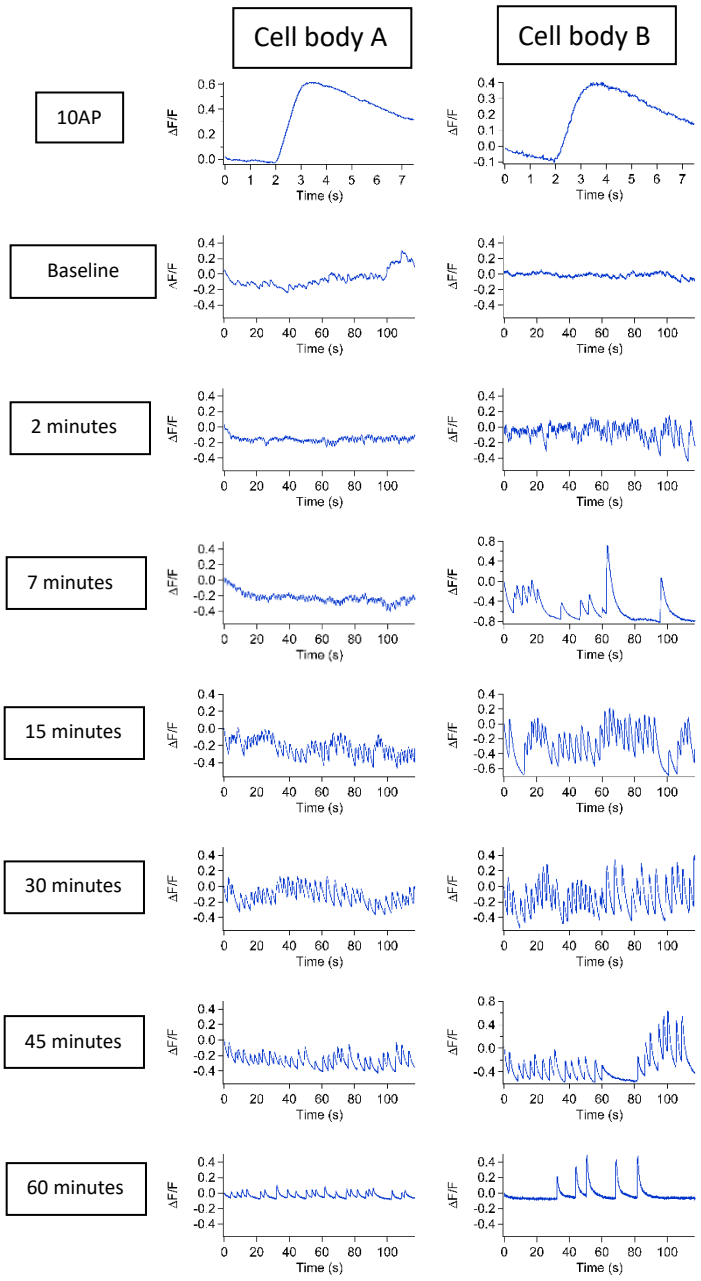


Figure 3.25- Overview of individual effect of 10µM of KV1.3 blocker compound on H2B-GCaMP6f. Representative examples of individual responses of two different side by side cell bodies (label as “Cell Body A” and “Cell Body B” on the figure) of CS1, in the absence of synaptic blockers. Each column corresponds to an individual cell body’s response over time.

In **Figure 3.24**, it is visible a different pattern response per coverslip. In CS1, it is visible the change on the shape of the responses two minutes after adding the compound, and then after seven minutes the response increased the amplitude of some single peaks. On 15, 30 and 45 minutes after the addition of the compound the response goes back to the initial pattern however with higher amplitudes. Finally, on 60 minutes after the addition of the compound the pattern totally change to five more sporadic peaks with not so high amplitude as the previous ones.

In CS2, there is no significant response to the addition of the compound, although it reacts to 10AP.

In CS3, the spontaneous activity changes once the compound is added by increasing the amplitude of the peaks, however in this coverslip the firing pattern does not change as much over time as CS1.

According to Figure 3.25, CS1 shows two different individual patterns over time as response to addition of 10µM KV1.3 blocker. Comparing the individual patterns with the final average of Figure 3.24, it is missing an important part of the individual responses.

H2B-GCaMP6f KV1.3 blocker 3µM NES++ **Averages**

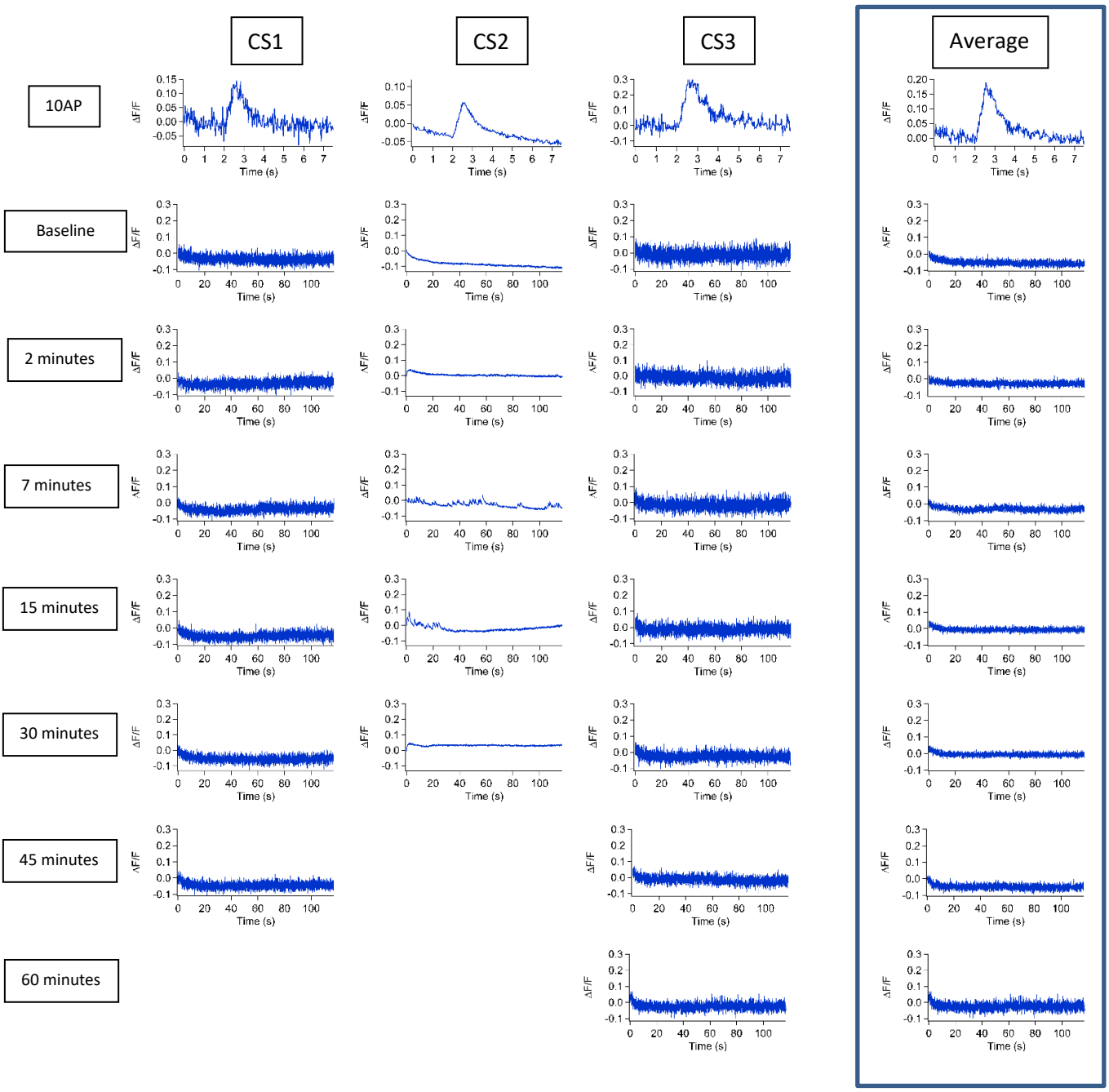


Figure 3.26- Overview of the average effect of 3µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 4 reacting cell bodies at DIV27, CS2 showed 2 reacting cell bodies at DIV14 and CS3 has 2 reacting cell body at DIV27. The total of cell bodies on this condition is 8.

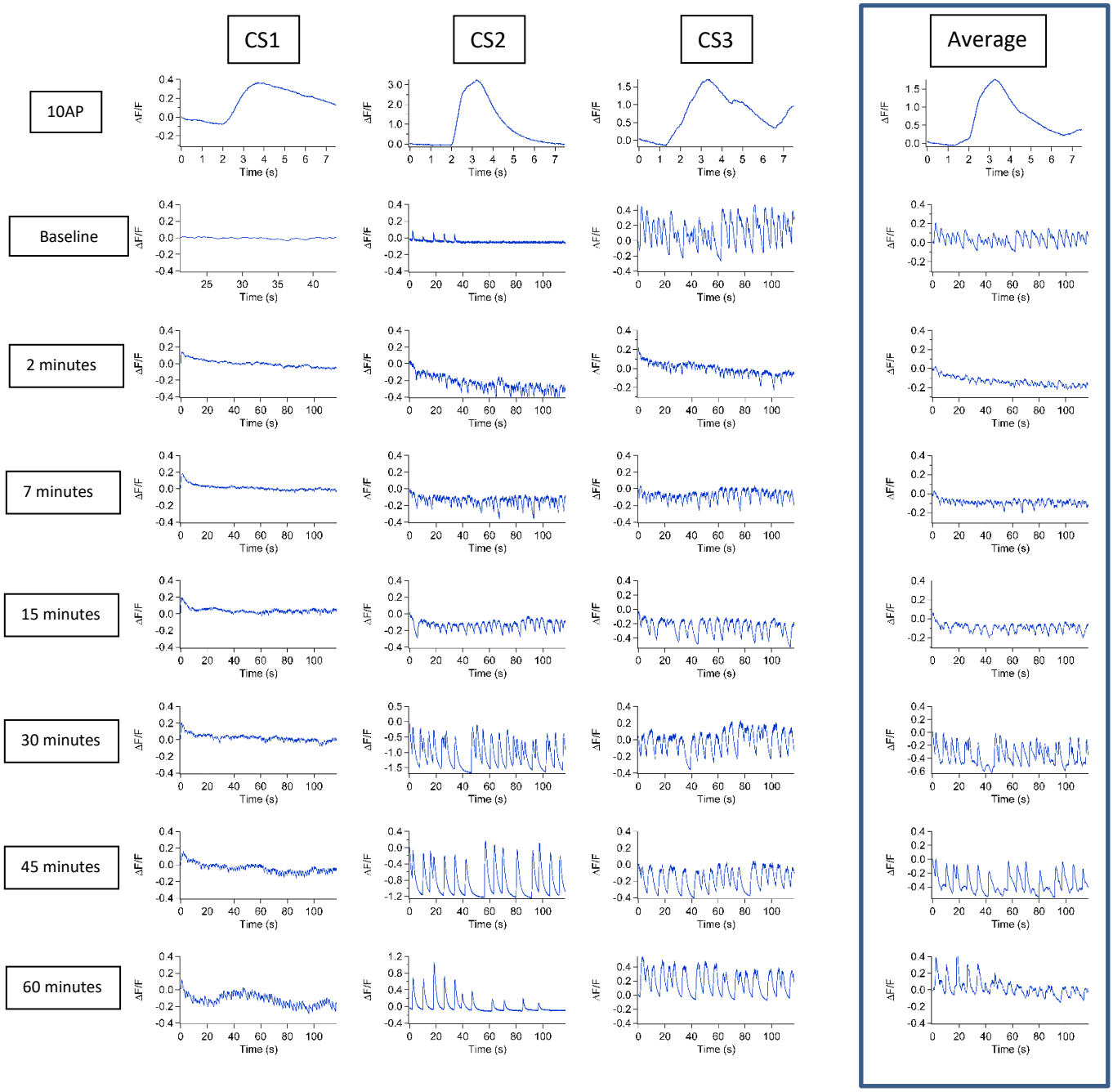


Figure 3.27- Overview of the average effect of 3µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 3 reacting cell bodies at DIV20, CS2 showed 3 reacting cell bodies at DIV14 and CS3 has 6 reacting cell body at DIV15. The total of cell bodies on this condition is 12.

H2B-GCaMP6f KV1.3 blocker 3µM NES **Representative examples** CS3

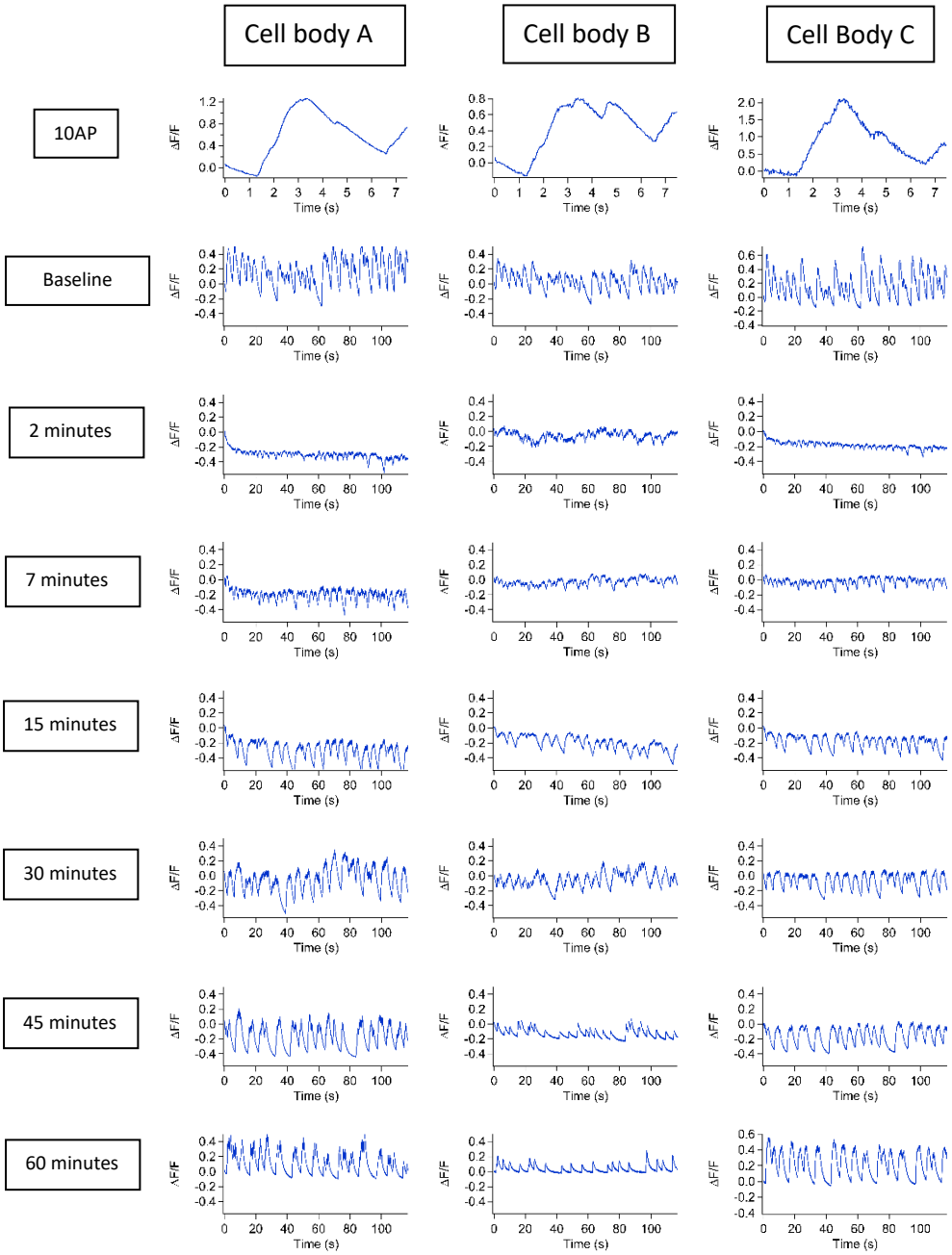


Figure 3.28- Overview of individual effect of 3µM of KV1.3 blocker compound on H2B-GCaMP6f. Representative examples of individual responses of three different side by side cell bodies (label as “Cell Body A”, “Cell Body B” and “Cell Body C” on the figure) of CS3, in the absence of synaptic blockers. Each column corresponds to an individual cell body’s response over time.

In **Figure 3.26**, none of the three coverslips reacts to 3µM of KV1.3 blocker in the presence of synaptic blockers, meaning that this concentration is not enough to change intrinsic membrane properties. Although, the same condition in the absence of synaptic blockers shows changes in the spontaneous activity (Figure 3.27).

In CS1 on **Figure 3.27**, the changes are not significant until 60 minutes after the addition of 3µM KV1.3 blocker, in which shows a slightly difference in amplitude comparing to the initial spontaneous activity. Although in CS2 the changes in the amplitude and firing pattern are clear – from 2 minutes until 15 minutes there are a small increase of the amplitude once the compound is added yet in minute 30 is visible that it gets progressively higher until minute 60 in which start to decrease significantly.
In CS3, there is a high spontaneous activity before and with the addition of the compound the spontaneous activity decrease significantly bur over time it returns to the initial pattern however with different frequency.

In **Figure 3.28**, the individual patterns follow a similar firing behavior until minute 15, then Cell Body A increases its firing amplitude and keep this pattern until minute 60. On the other hand, Cell Body B change the shape of the peaks turning them less broad until it shows a completely different firing mode on minutes 45 and 60. Cell Body C shows similar response until minute 45 and then at minute 60 there is a slightly difference on the amplitude of the firing pattern.

In **Figure 3.29,** beside two sporadic and small peaks in two different coverslips – minute 45 of CS2 and minute45 on CS3-, the general idea is that 1µM of KV1.3 blocker does not do significant changes on intrinsic membrane properties.

On the other hand, in **Figure 3.30**, in the absence of synaptic blockers, 1µM of KV1.3 blocker alters the firing pattern of the cells. As shown in Figure 3.30, CS1 does not show significant changes in the firing pattern, however CS2 starts with an abnormal spontaneous activity even being in the absence of synaptic blockers. Later, it shows alterations only on minute 7 – small peaks on spontaneous activity- and after the signal starts to increase drastically at minute 15, keeping the maximum intensity - which is much higher than 10AP response - for almost 50 seconds. Then from minute 15 until minute 60 the firing amplitude stays higher than 10AP until the last half of minute 60's recording, where it decreases drastically again. This can be a behavior of a cell body dying or under an extreme stress.
In CS3, the cell has a very strong and not normal spontaneous activity even before the compound, which completely gets smoother and less broad once the compound is added. Until minute 60 the pattern is similar over time, but in minute 60 the pattern of firing change and turning into to several clearer peaks.
To analyze cell bodies' individual patterns in more detail, in **Figure 3.31**, CS1 was chosen since it is the one that shows a normal responsive behavior, between the three coverslips. It is possible that changes in the average response of CS1 are due to the pattern of only one cell body of three, because the other two coverslips do not respond with significant changes to 1µM of KV1.3 blocker.

H2B-GCaMP6f KV1.3 blocker 1µM NES++ **Averages**

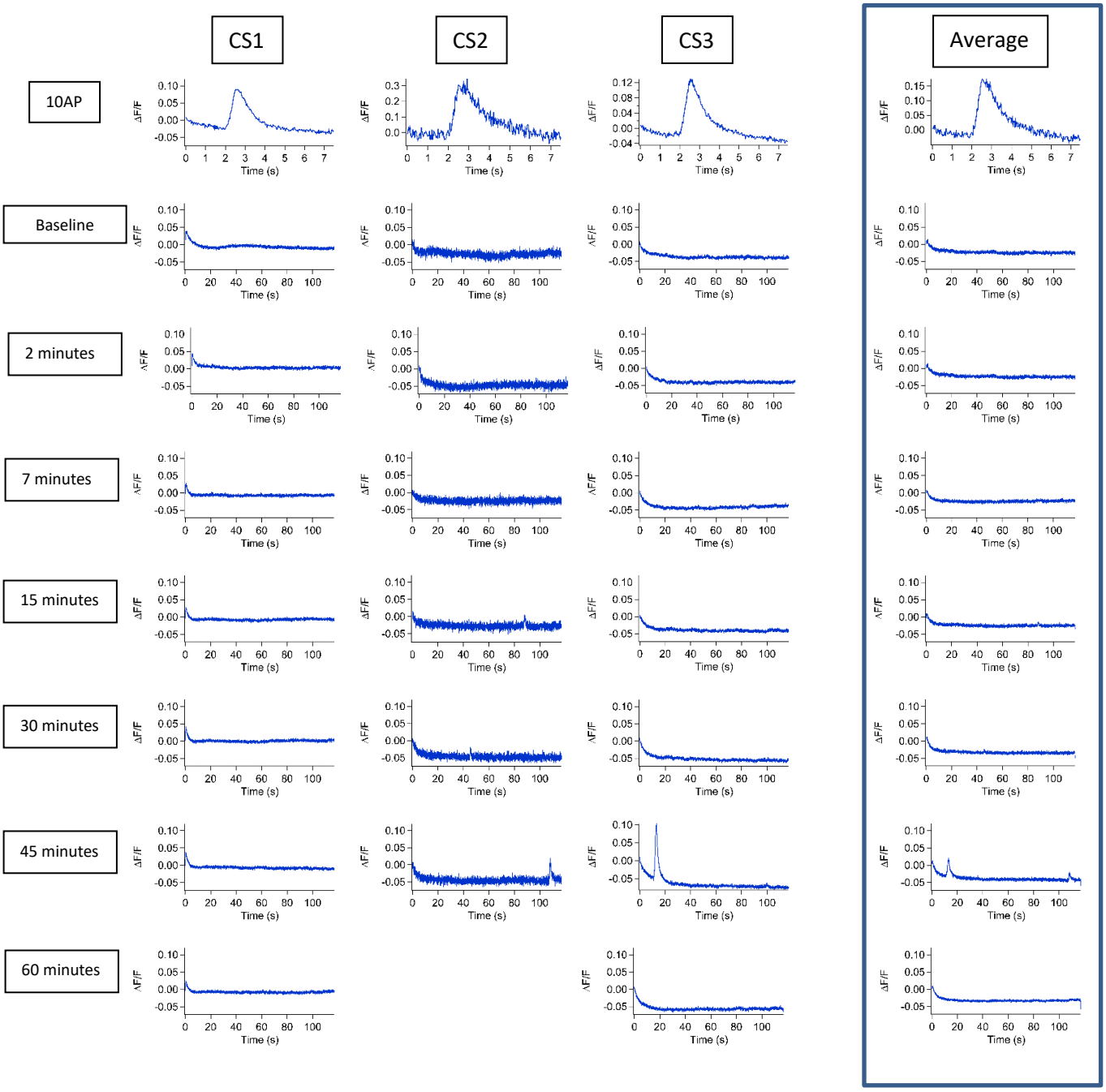


Figure 3.29- Overview of the average effect of 1µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 3 reacting cell bodies at DIV20, CS2 showed 3 reacting cell bodies at DIV15 and CS3 has 3 reacting cell body at DIV19. The total of cell bodies on this condition is 9.

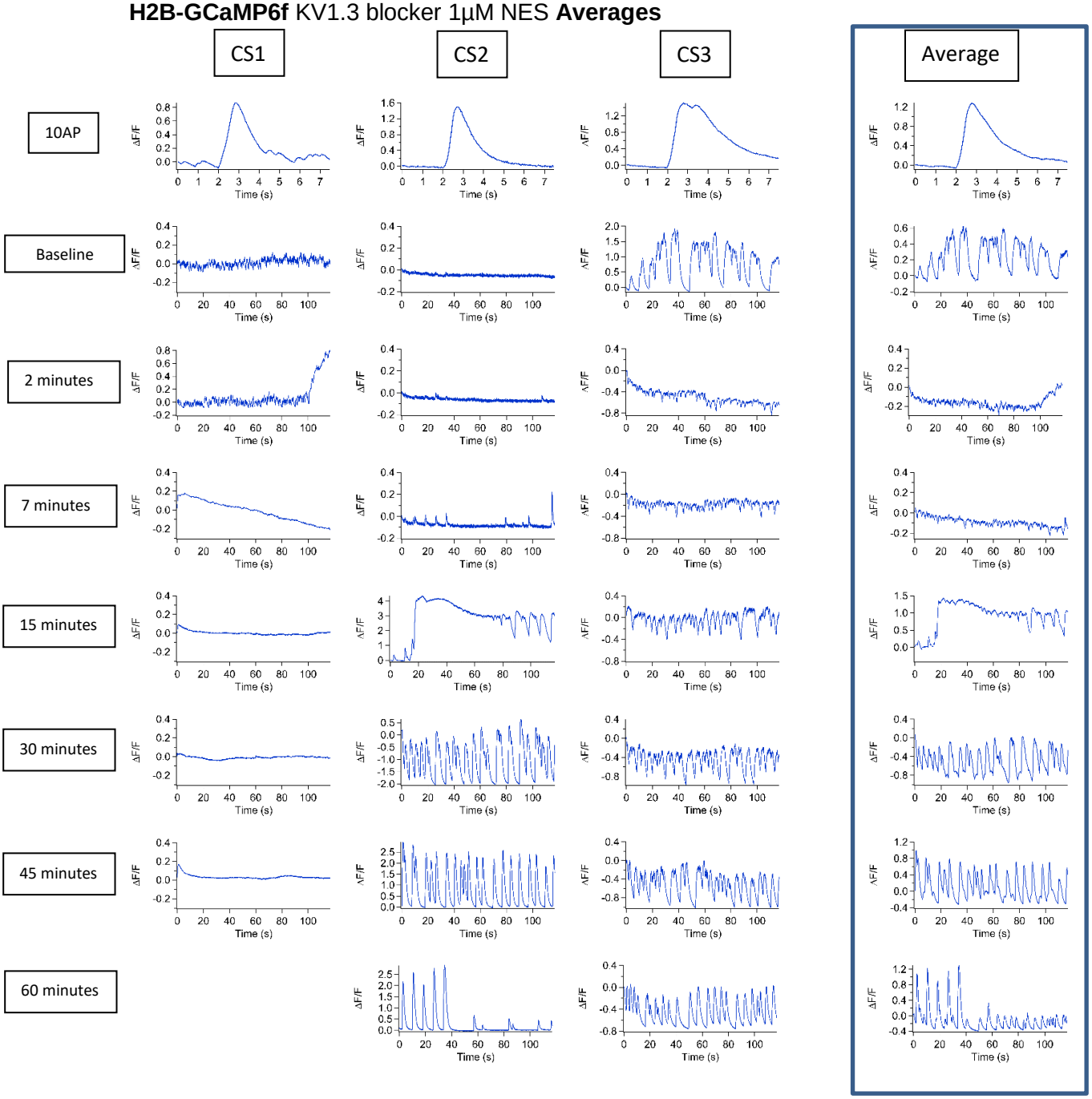


Figure 3.30- Overview of the average effect of 1µM of KV1.3 blocker compound on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 3 reacting cell bodies at DIV23, CS2 showed 2 reacting cell bodies at DIV14 and CS3 has 4 reacting cell body at DIV15. The total of cell bodies on this condition is 9.

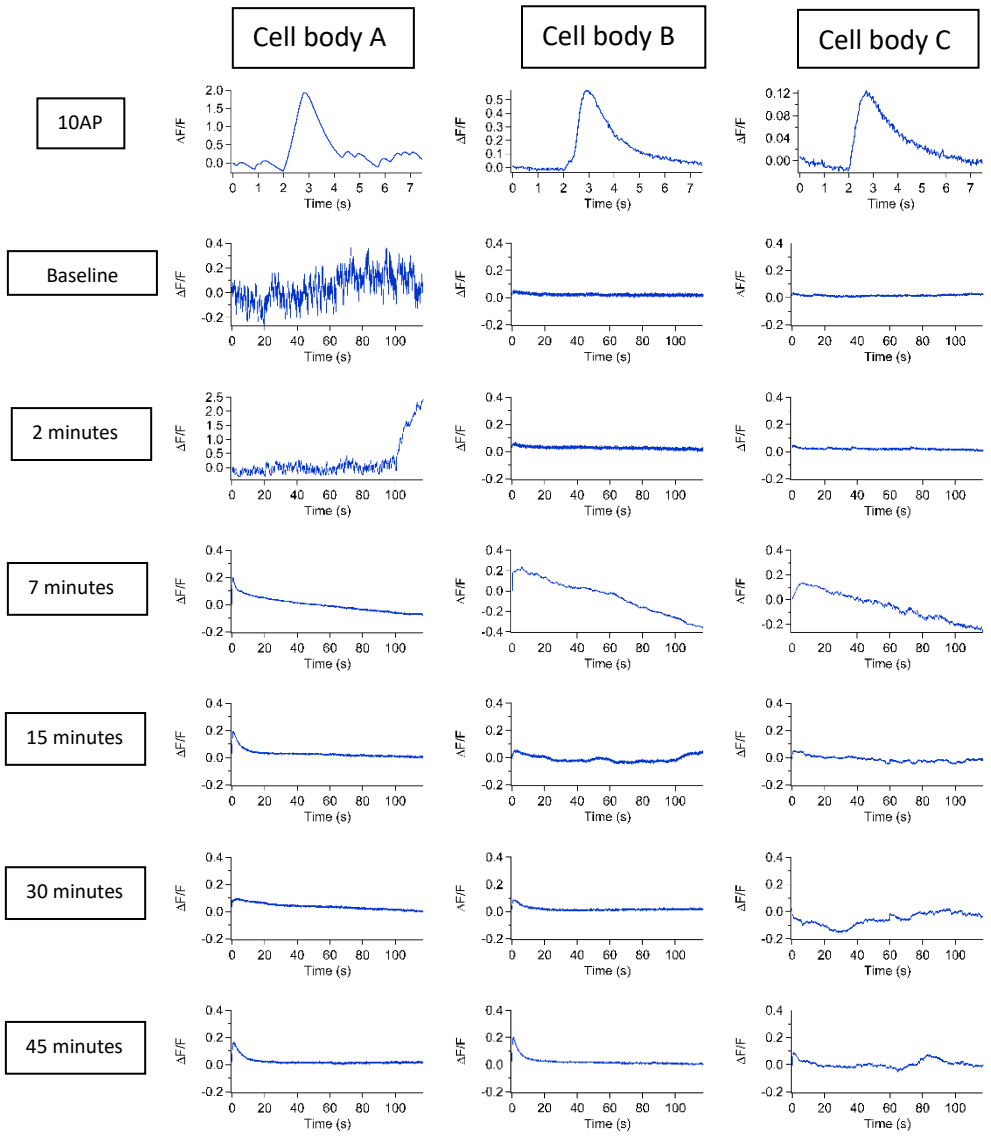


Figure 3.31- Overview of individual effect of 1µM of KV1.3 blocker compound on H2B-GCaMP6f. Representative examples of individual responses of three different side by side cell bodies (label as “Cell Body A”, “Cell Body B” and “Cell Body C” on the figure) of CS1, in the absence of synaptic blockers. Each column corresponds to an individual cell body’s response over time.

SyGCaMP6f

To compare nuclear responses with synaptic responses, the same conditions – 10µM in NES++, 10µM in NES, 3µM in NES++, 3µM in NES, 1µM in NES++ and 1µM in NES were performed but with SyGCaMP6f instead of H2B-GCaMP6f.

In **Figure 3.32,** it is visible that at least one coverslip -CS2- reacts to 10µM of KV1.3 blocker with a firing pattern completely different from the one without compound on. Synapses of CS2 show a normal spontaneous activity in the presence of blockers however once the compound was added the cells started firing and the firing pattern started to get slightly broader peaks until minute 30. At minute 30 the firing pattern does not show peaks anymore however in minute 45 small peaks appeared again in spontaneous activity.

CS1 does not show any significant change in spontaneous activity over time with the addition of the compound but it also shows small peaks when there is no compound on yet those small peaks disappeared over time. Additionally, CS3 shows two small peaks in minute 2 but in general there is no significant response over time.

In **Figure 3.33**, there are three different examples of synaptic responses in the presence of synaptic blockers to 10µM of the compound. Synapses from the same region in the same coverslip (CS2). While Synapse A reacts with single peaks which are constant in minute 2 but then they become more sporadic until they disappeared in minute 15, Synapse B shows smaller peaks in minute 2 and then they become more constant in minute 7 comparing with Synapse A. Additionally Synapse B's peaks last longer, showing two sporadic peaks at minute 15. On the other hand, Synapse C shows a significant response over time, although the response is more frequent in the beginning – minute 2 and 7. Then it starts to get less frequent on the firing rate and to show broader peaks, until minute 60 where the peaks are not clear anymore. Comparing the three-different pattern existing in CS2 with the average of the same, there are some patterns not shown clearly

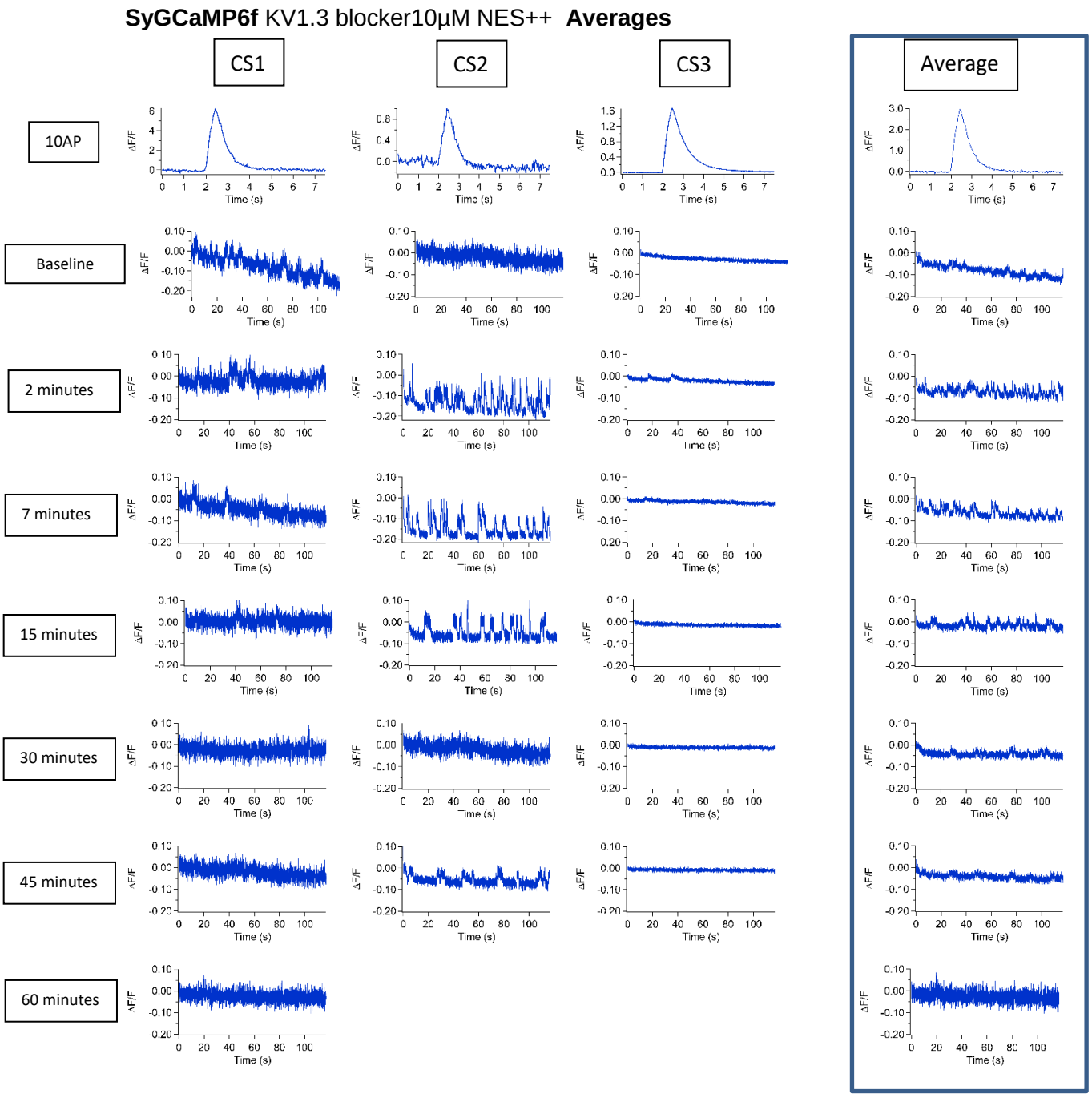


Figure 3.32- Overview of the effect of 10µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 shows the average response of 47 reacting synapses at DIV 20, CS2 shows the average of 143 reacting synapses at DIV15 and CS3 shows the average response of 97 reacting synapses at DIV19. The total of reactive synapses on this condition is 287.

SyGCaMP6f KV1.3 blocker10µM NES++ **Representative examples CS2**

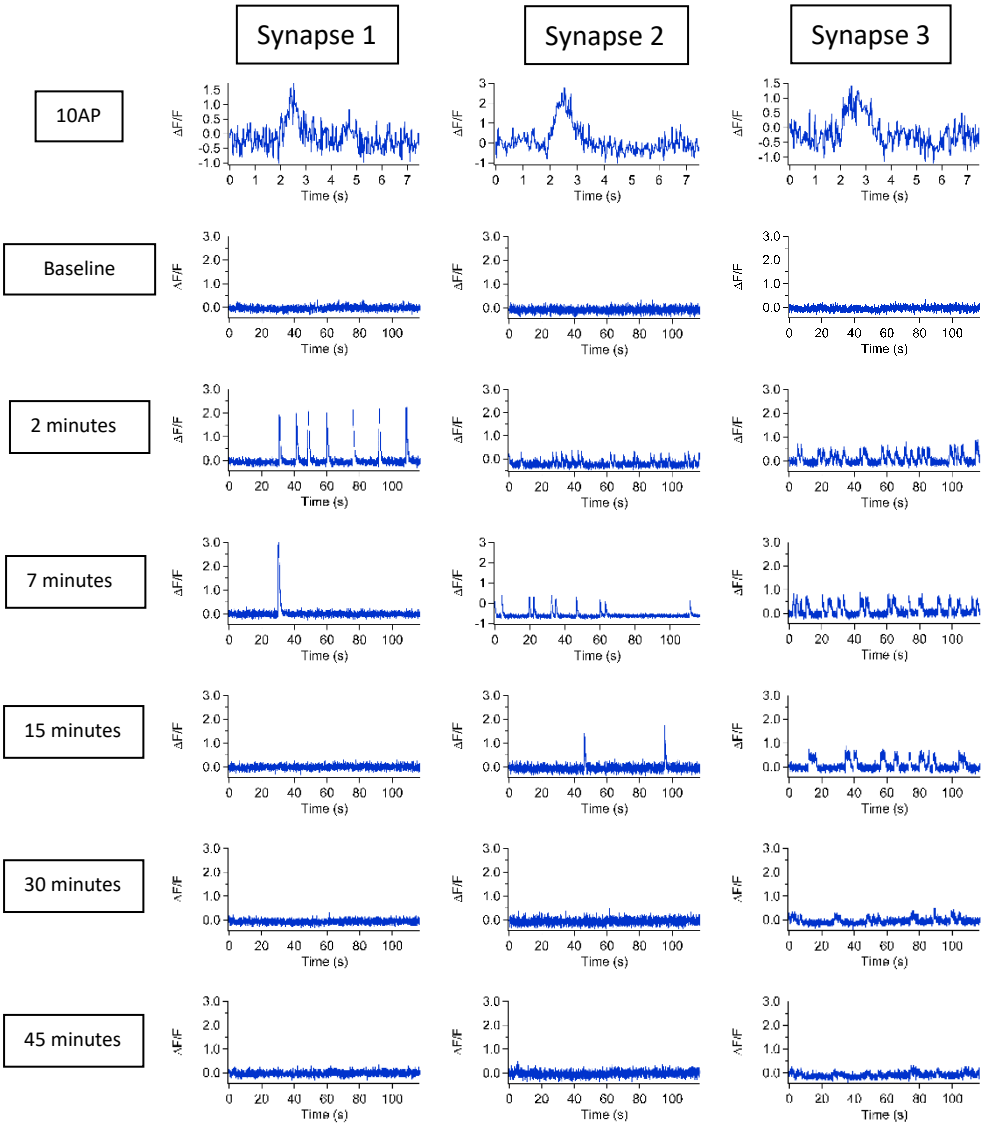


Figure 3.33- Overview of individual effect of 10µM of KV1.3 blocker compound on SYGCaMP6f. Representative examples of individual responses of three different side by side synapses (label as “Synapse 1”, “Synapse 2” and “Synapse 3” on the figure) of CS2, in the presence of synaptic blockers. Each column corresponds to an individual synapse’s response over time. In CS2, 45 synapses from 143 (31.4%) exhibit the pattern of synapse 1, while 49 synapses from 143 (34%) exhibit the pattern of synapse 2. 27% of 143 reactive synapses do not respond to the compound and the 7,6% left show different patterns which are not similar to those shown on the figure.

At this point it is already possible to understand that synapses are much more sensitive to 10µM of the compound than H2B-GCaMP6f (see more details in Discussion).

The next step is to study the same conditions but in absence of synaptic blockers.
The results of the next batch of experiments showed sometimes a spontaneous activity too spiky becoming unclear to analyze. In cases of too spiky spontaneous activity it was necessary to zoom in a random range between 40 and 60 seconds to see in more detail the firing pattern.

In **Figure 3.34.A**, CS1 shows different patterns over time. First, in minute 2 it shows a change in the frequency, turning it to a spikier response. In minute 7, there is a change on the shape of the peak becoming broader and less spiky than before. Later, on minute 15, it got spiky again, and from then on the response become less spiky progressively.
In CS2, the response started to appear on minute 7 with small peaks, then in minute 15 the peaks got less clear and on minute 30 the amplitude of the firing increases drastically as well as in minute 45.

In **Figure 3.34.B** CS3 shows a small spontaneous activity although with the formatted scale the fluctuations are not clear. In addition, it is the coverslip with less amplitude of 10AP response meaning that the synapses of that coverslip could be less reactive than the others. There are sporadic and small peaks happening until minute 15, and disappearing from then on.

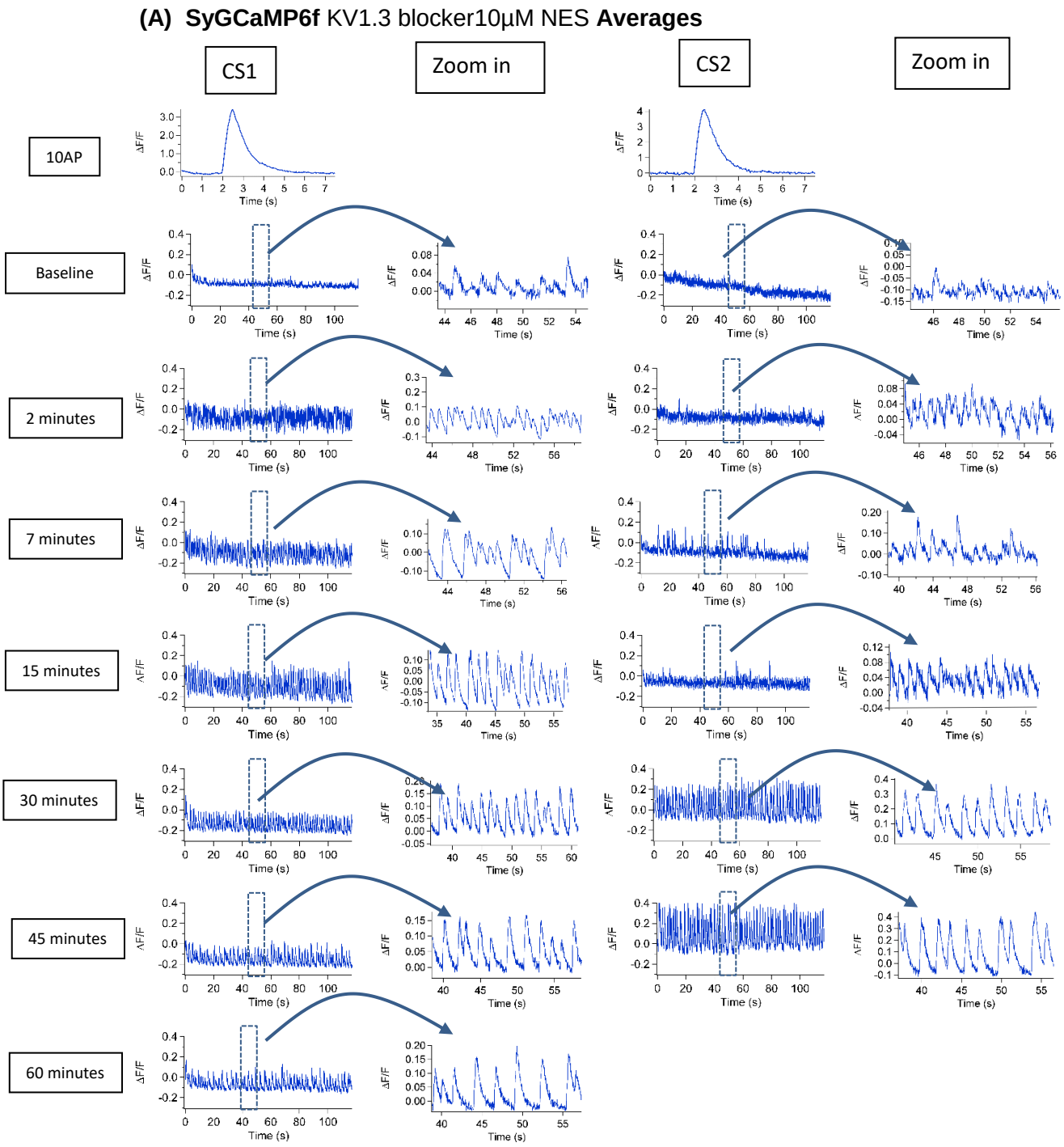


Figure 3.34.A- Overview of the effect of 10µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the absence of synaptic blockers. The first and third columns show the average of the responses over time per coverslip: Coverslip 1 (CS1) and Coverslip 2 (CS2), and the second and last column are showing the corresponding zoom in the selected area of the graph.CS1 shows the average response of 32 reacting synapses at DIV26, CS2 shows the average of 34 reacting synapses at DIV28.

(B) SyGCaMP6f KV1.3 blocker10µM NES **Averages**

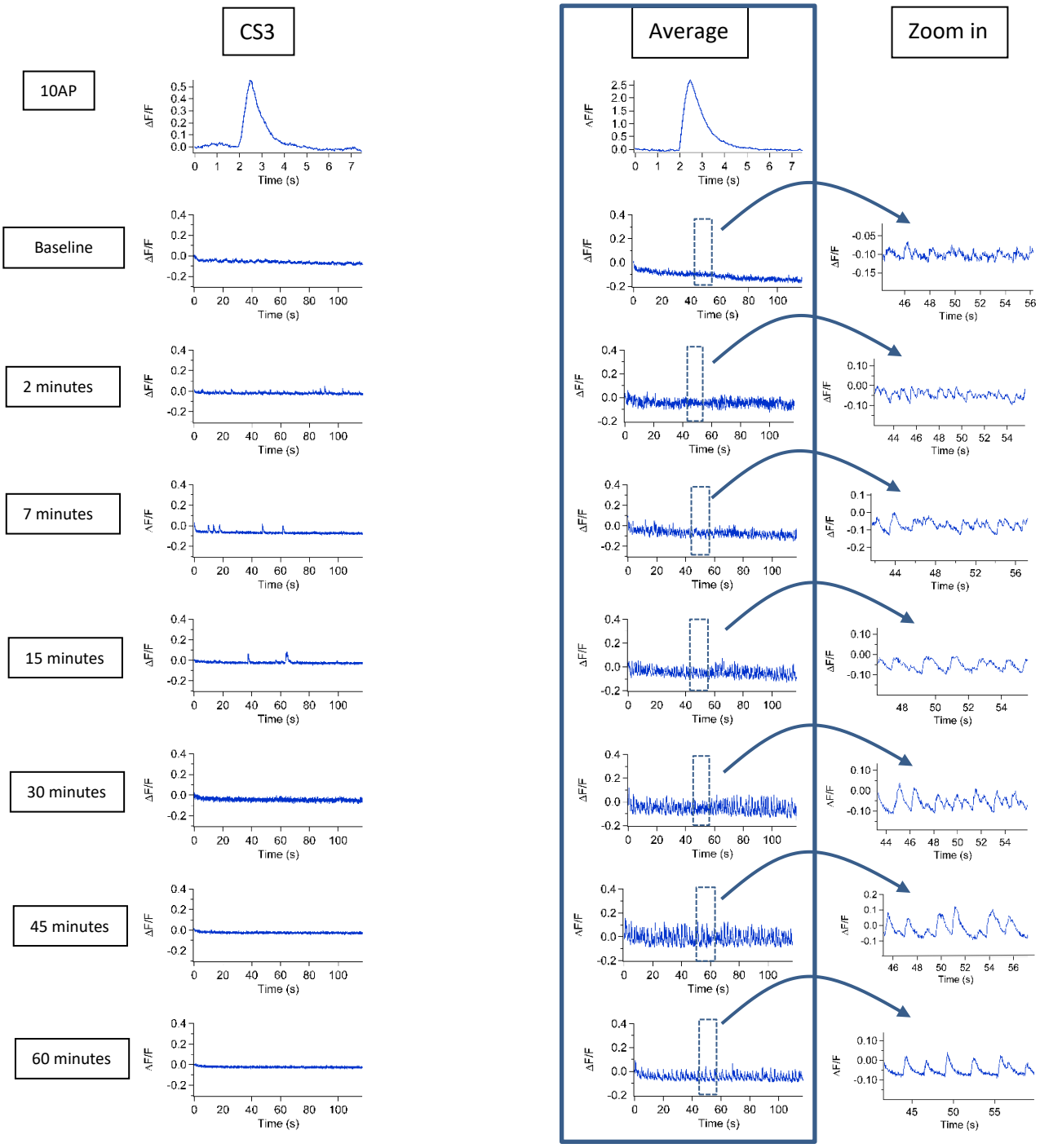


Figure 3.34.B- Overview of the effect of 10µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the absence of synaptic blockers. The first column shows the average of the response over time of CS3. The second column is the average of all coverslips for this condition (n=3) while the third column is the respective zoom in of the selected area on the graph. CS3 shows the average response of 126 reacting synapses at DIV19. The total of reactive synapses on this condition is 192.

SyGCaMP6f KV1.3 blocker10µM NES **Representative examples**

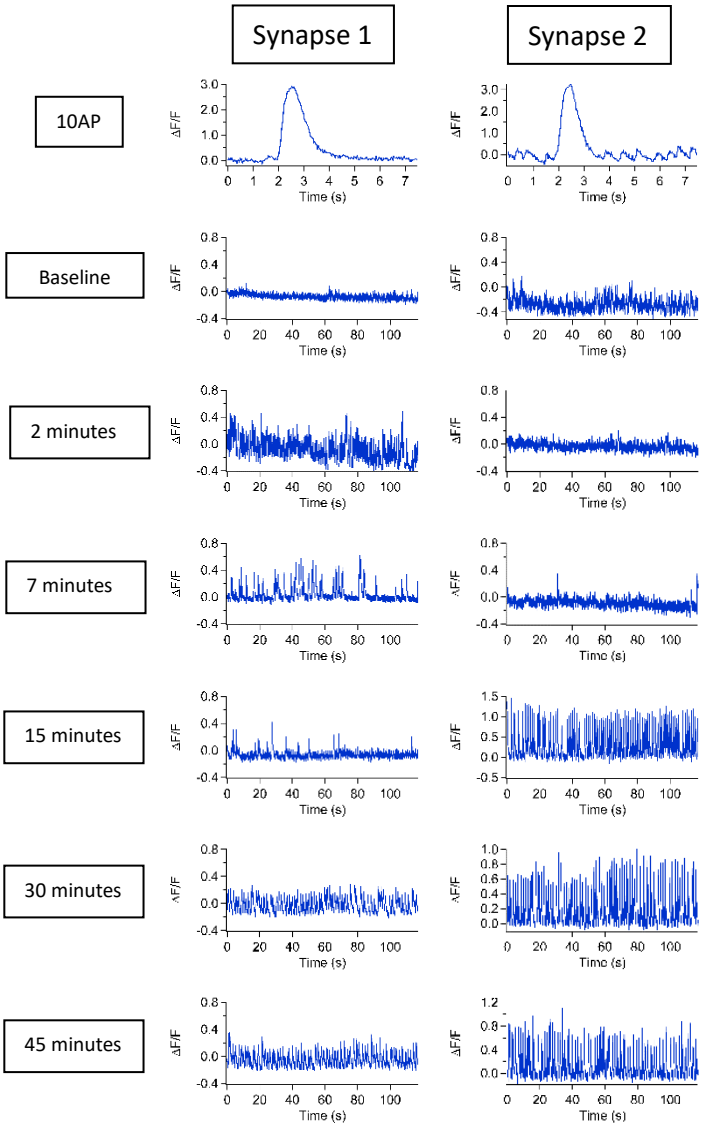


Figure 3.35- Overview of individual effect of 10µM of KV1.3 blocker compound on SyGCaMP6f, in the absence of synaptic blockers. Representative examples of individual responses of two different synapses (label as “Synapse 1” and “Synapse 2” on the figure) of CS2. Each column corresponds to an individual synapse’s response over time. In CS2, 4 synapses from 34 (11,7%) exhibit the pattern of synapse 1, while 5 synapses from 34 (14,7%) exhibit the pattern of synapse 2. The other synapses do not exhibit the same pattern as a response to the compound or do not respond at all.

In **Figure 3.35**, it is shown two different patterns of response from synapses in the same region and same conditions. Synapse A looks like it is reacting first to the compound by turning the spontaneous activity less noisy and showing more clear spikes until minute 15. From minute 15, the signal becomes spikier until minute 45. Meanwhile synapse B looks like it took more time to react to the compound and when it did the response was a drastic boost on firing amplitude and rate, from minute 15 to minute 45.

Knowing that 10µM of KV1.3 blocker induces changes in the synapses in the presence and absence of synaptic blockers, it was tested if 3µM of the same compound could induce similar changes.

Figure 3.36 answers the hypothesis proposed above showing that 3µM does not induce any change on spontaneous activity in the presence of synaptic blockers, however in the absence of synaptic blockers it does (Figure 3.37).

In **Figure 3.37**, CS1, the spontaneous activity with no compound is strong and it decrease radically two minutes after the addition of the compound. Then it is clear that the amplitude of the firing increases although does not reach the initial amplitude. It is important to say that CS1's response to 10 AP is abnormally reactive as well.
In CS2, a small change in the amplitude happens from minute 15 to minute 45 but it not as significant as CS1. In CS3, the spontaneous activity is not normal for the condition although it is noticeable that the 3µM of compound changes drastically the firing mode over time, specifically on 15 minutes.

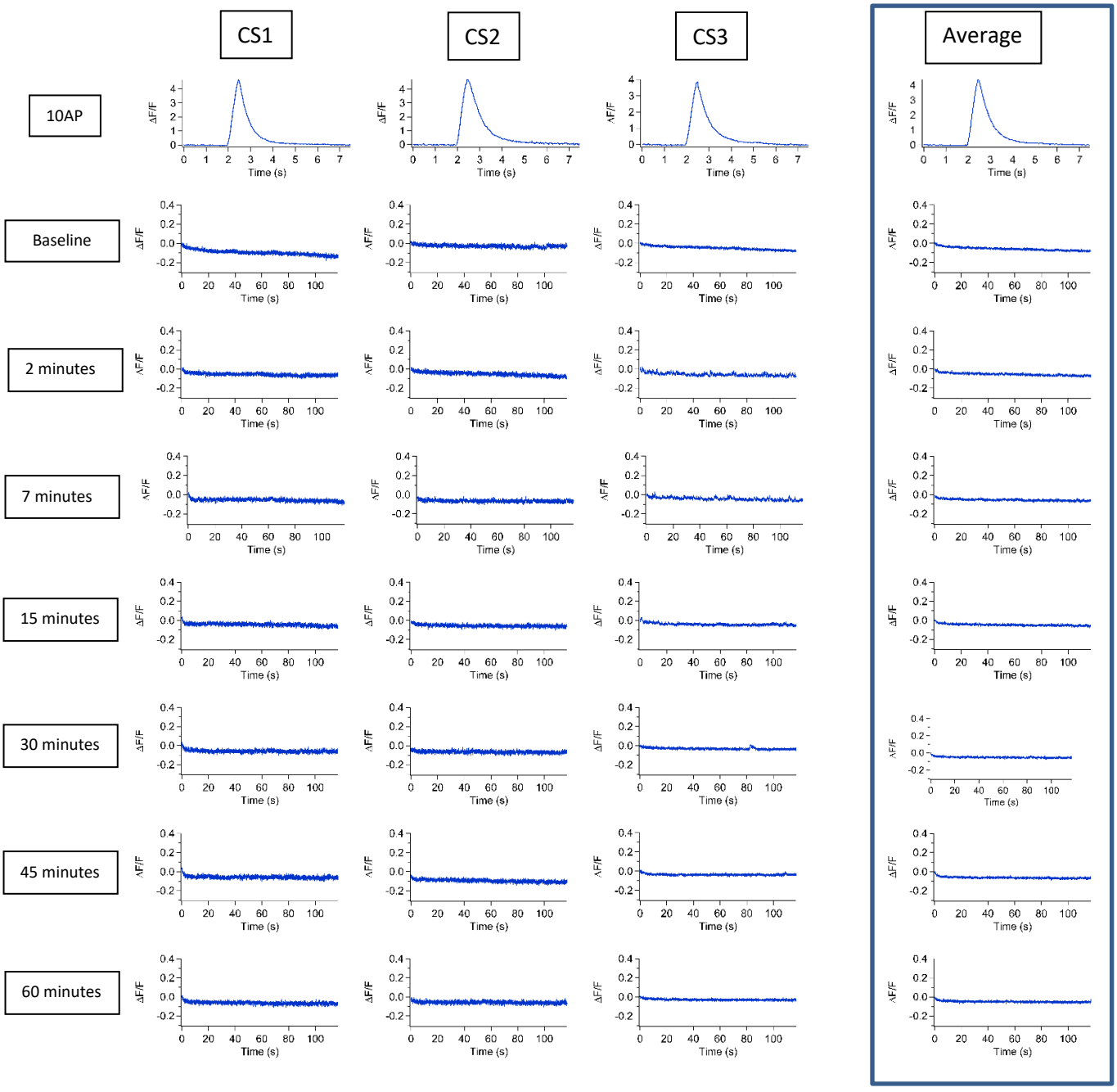


Figure 3.36- Overview of the effect of 3µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 shows the average response of 25 reacting synapses at DIV 27, CS2 shows the average of 54 reacting synapses at DIV14 and CS3 shows the average response of 44 reacting synapses at DIV22. The total of reactive synapses on this condition is 123.

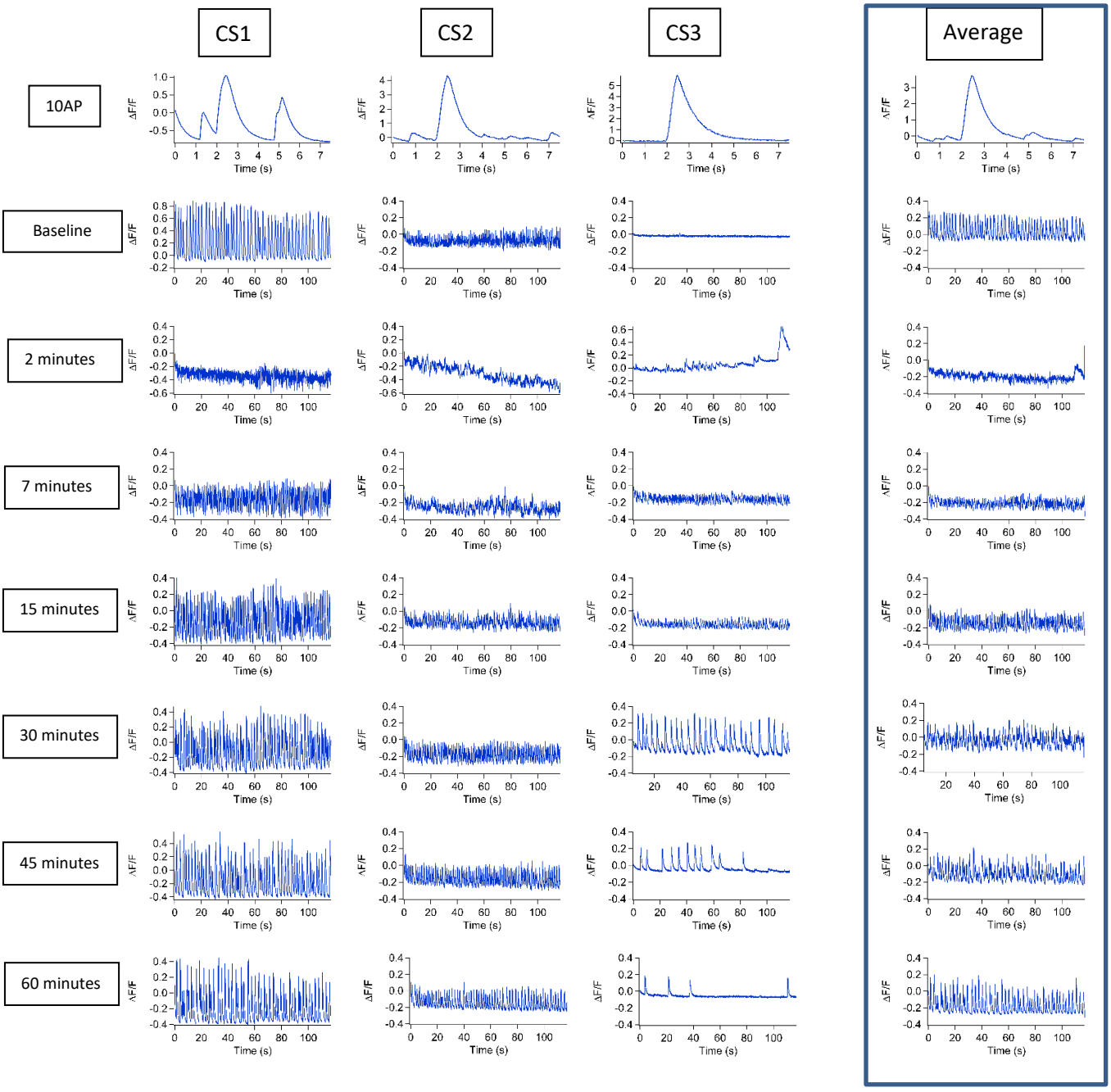


Figure 3.37- Overview of the effect of 3µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 shows the average response of 27 reacting synapses at DIV 26, CS2 shows the average of 36 reacting synapses at DIV26 and CS3 shows the average response of 109 reacting synapses at DIV19. The total of reactive synapses on this condition is 172.

SyGCaMP6f KV1.3 blocker 3µM NES **Representative examples CS2**

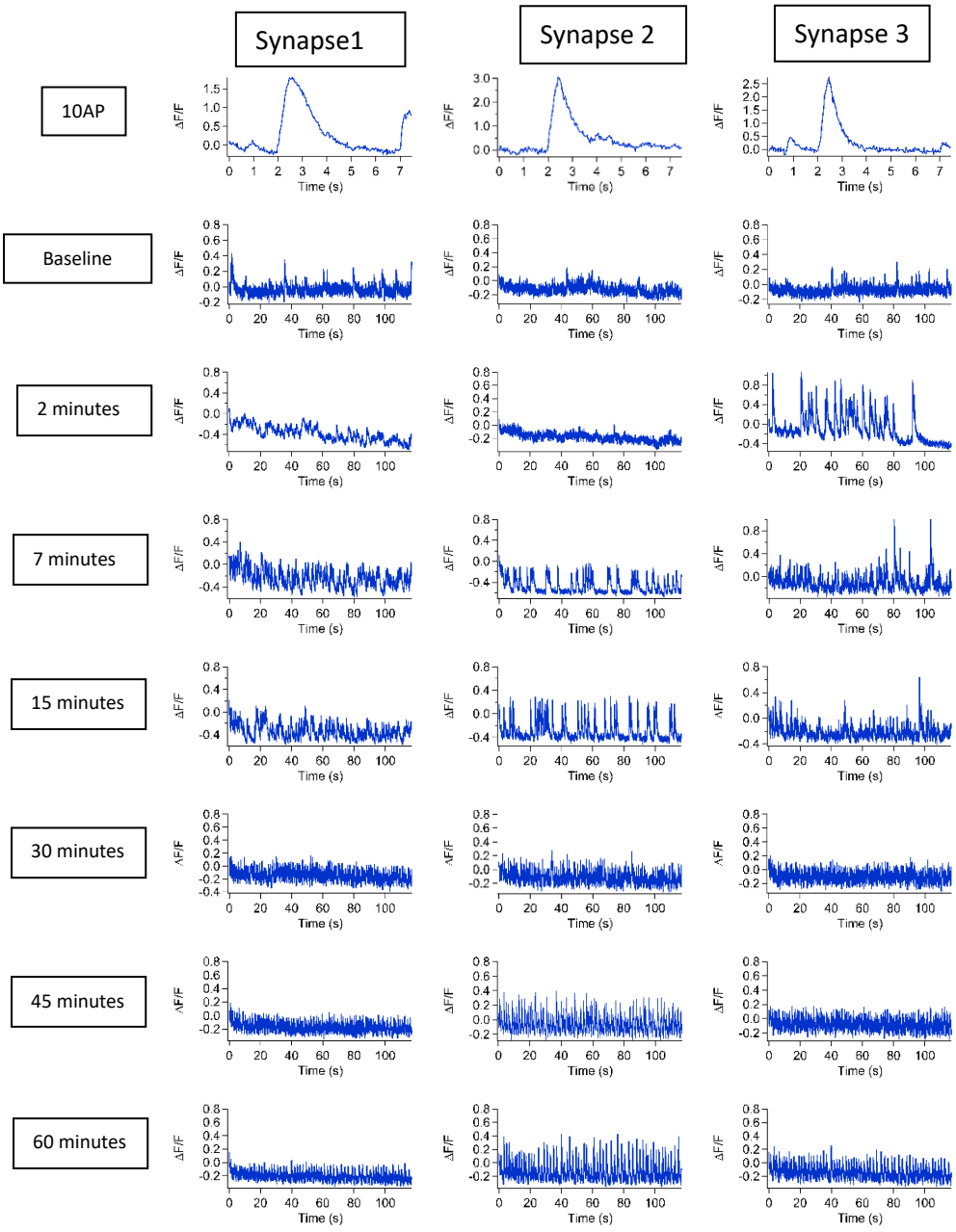


Figure 3.38- Overview of individual effect of 3µM of KV1.3 blocker compound on SYGCaMP6f, in the absence of synaptic blockers. Representative examples of individual responses of three different side by side synapses (label as “Synapse 1”, “Synapse 2” and “Synapse 3” on the figure) of CS2. Each column corresponds to an individual synapse’s response over time. In CS2, 16 synapses from 34 (47%) exhibit the pattern of synapse 1, while 6 synapses from 34 (17,6%) exhibit the pattern of synapse 2, and 12 synapses from 34 (35,3%) reactive synapses show pattern of synapse 3.

In **Figure 3.38**, Synapse 1 shows a decrease of number of spikes as a first reaction to the compound at minute 2, although in minute 7 there is a boost on firing rate that stays until 15 minutes and after that it starts to be spikier until minute 60.
Synapse 2, starts to show clear and constant spikes at minute 7 and minute 15 but then at minute 30 the spikes are not as clear as before. At minute 45 the pattern of firing changes drastically to a broader and less spikes pattern, and then at minute 60 the same pattern of minute 15 comes back but much spikier.
In Synapse 3 a significant response happens to minute 2 with peaks of high amplitude that diminish the frequency but not the amplitude until minute 30. From minute 30 to minute 45 spikes are not visible anymore however in minute 60 it is clear a response showing more spikes and having the same amplitude as the initial spontaneous activity.

The next step was to study the concentration of 1µM in synaptic responses, and **Figure 3.39** shows that this concentration does nothing in the presence of synaptic blockers.

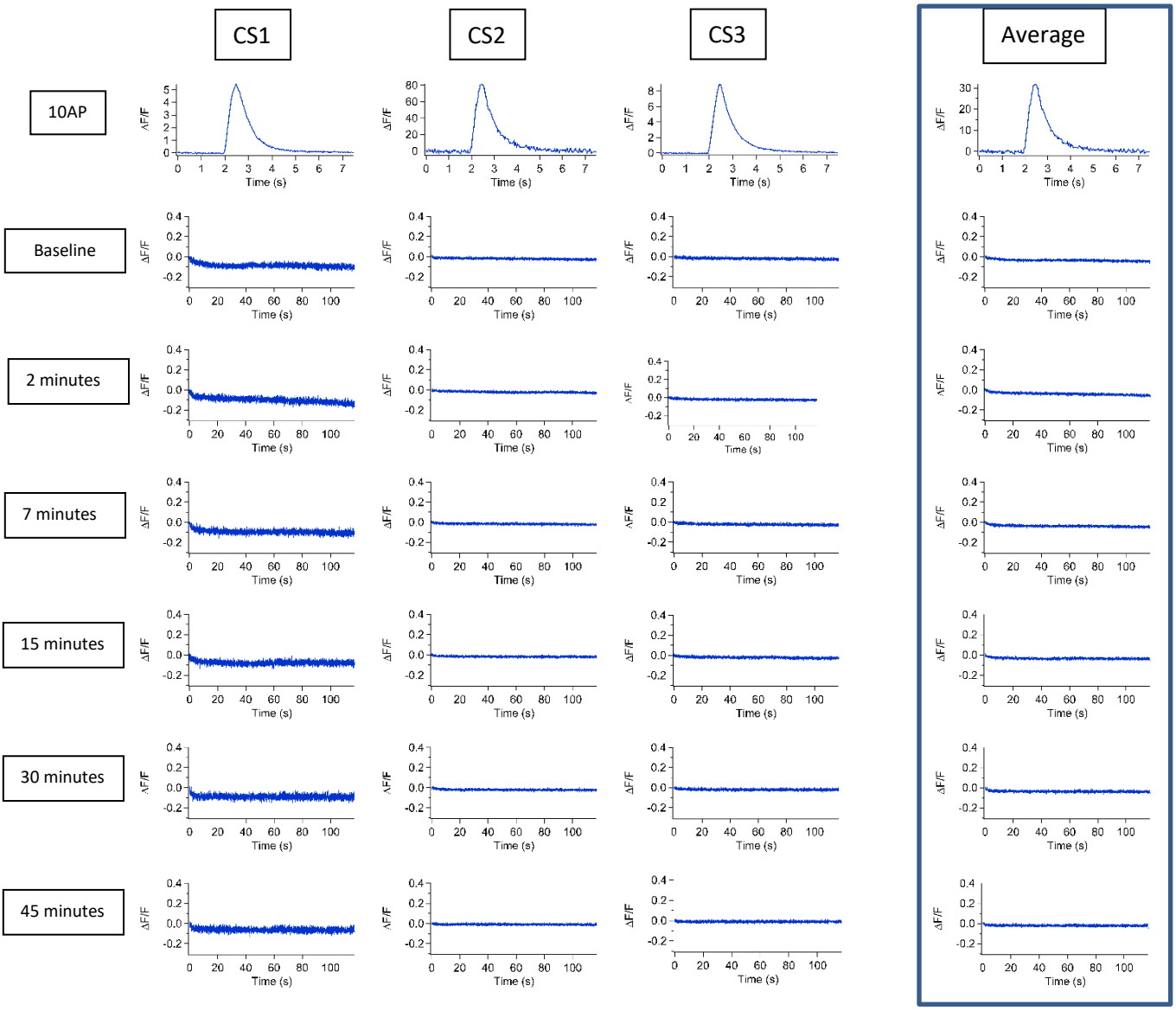


Figure 3.39- Overview of the effect of 1µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 shows the average response of 13 reacting synapses at DIV 27, CS2 shows the average of 19 reacting synapses at DIV20 and CS3 shows the average response of 18 reacting synapses at DIV20. The total of reactive synapses on this condition is 50.

SyGCaMP6f KV1.3 blocker 1µM NES **Averages**

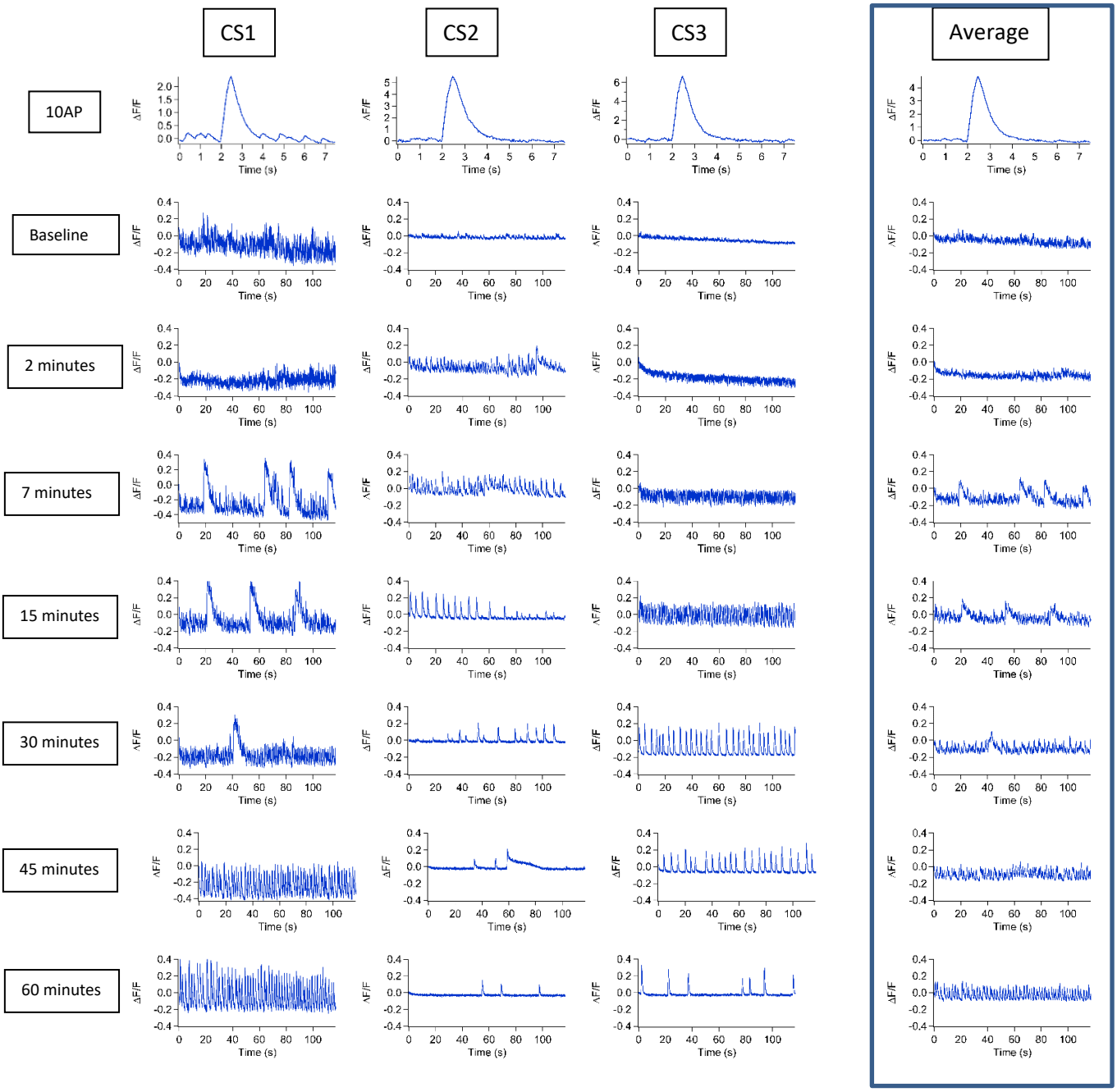


Figure 3.40- Overview of the effect of 1µM of KV1.3 blocker compound on SYGCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 shows the average response of 6 reacting synapses at DIV 26, CS2 shows the average of 57 reacting synapses at DIV20 and CS3 shows the average response of 88 reacting synapses at DIV20. The total of reactive synapses on this condition is 151.

SyGCaMP6f KV1.3 blocker 1µM NES **Representative examples CS1**

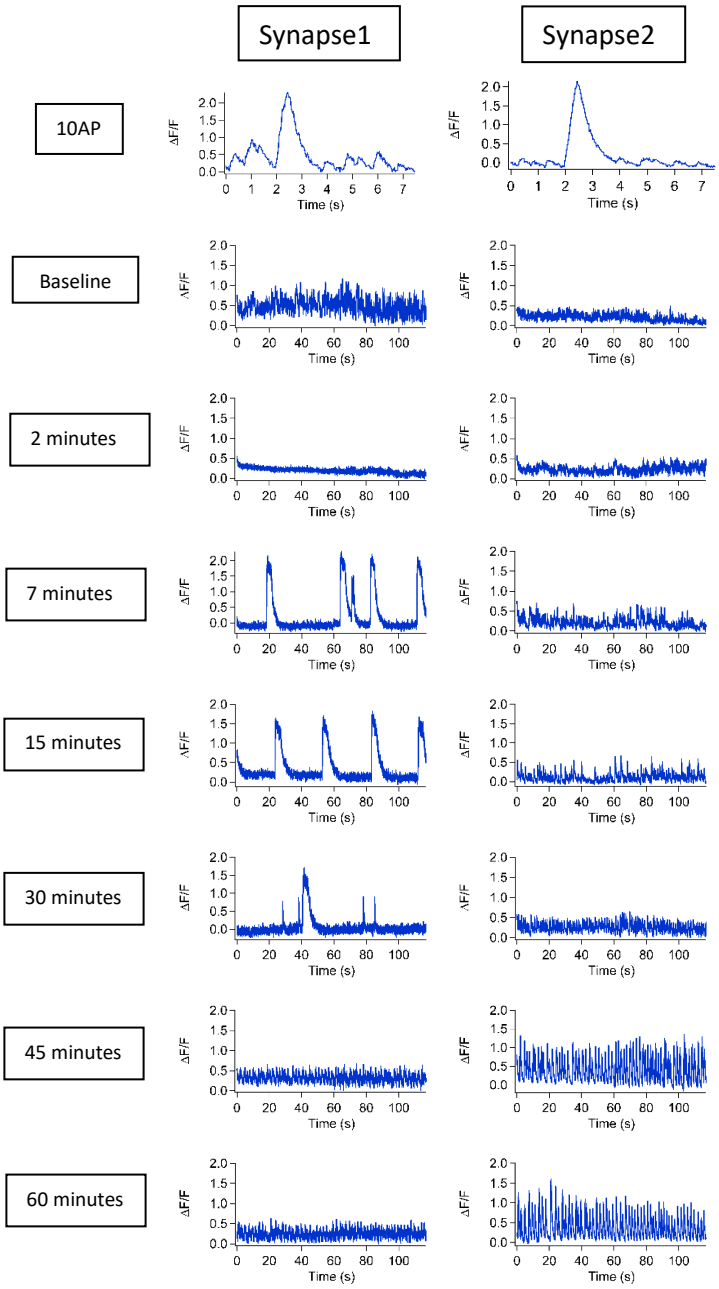


Figure 3.41- Overview of individual effect of 1µM of KV1.3 blocker compound on SYGCaMP6f, in the absence of synaptic blockers. Representative examples of individual responses of three different side by side synapses (label as “Synapse 1” and “Synapse 2” on the figure) of CS1. Each column corresponds to an individual synapse’s response over time. In CS1, 2 synapses from (33,3%) exhibit the pattern of synapse 1, while 4 synapses from (66,6%) exhibit the pattern of synapse 2.

In **Figure 3.40**, CS1 started to show peaks after 7 minutes of the addiction of the compound, keeping the pattern until minute 30 in which instead of three broad spikes it just shows one broad spike. At minute 45 the response becomes spiky and constant and it goes until minute 60.

In **Figure 3.41**, it is shown two representative examples in which it is noticeable, by the amplitude of synapse 1 and Synapse 2 on minute 7 that spikes from synapse 1 are much larger than the spikes on synapse 2. On minute 15, the pattern of both looks more constant and then at minute 30 synapse 1 shows a large spike and synapse 2 shows no visible spikes. After that synapse 1 does not show more spikes while synapse 2 shows a constant and more frequent firing rate until minute 60.

Compound X

This compound is from a phenotypic screening effort within J&J. It is called Compound X due to confidentiality reasons. For the next compound the concentration used was 10 µM in NES++, and then, after four coverslips with no responses, 10 µM, 3 µM and 1 µM was tested in NES. Only H2B-GCaMP6f experiments were performed.

It is important to refer that there is no presentation of representative examples because all the cell bodies showed similar patterns with this compound, so the average of each coverslip represents clearly the only pattern on it.

Figure 3.42 shows that no coverslip from the four coverslips tested reacts to 10µM of compound X in the presence of synaptic blockers, although in the absence of synaptic blockers it shows significant changes, as shown in Figure 3.43.

CS1 from **Figure 3.43** shows an increase of amplitude and firing rate until minute 15, and then at minute 30 it just shows two spikes which disappear until minute 60 in which shows no spike at all. Meanwhile, CS2 does not react to Compound X, although it reacts to 10AP. CS3 shows no significant responses until 30 minutes after the addiction of the compound and then at minute 30 there is an extreme increase of the amplitude- even above 10AP response's amplitude- which repeats at minute 45 and the stop at minute 60.

H2B-GCaMP6f Compound X 10µM NES++ **Averages**

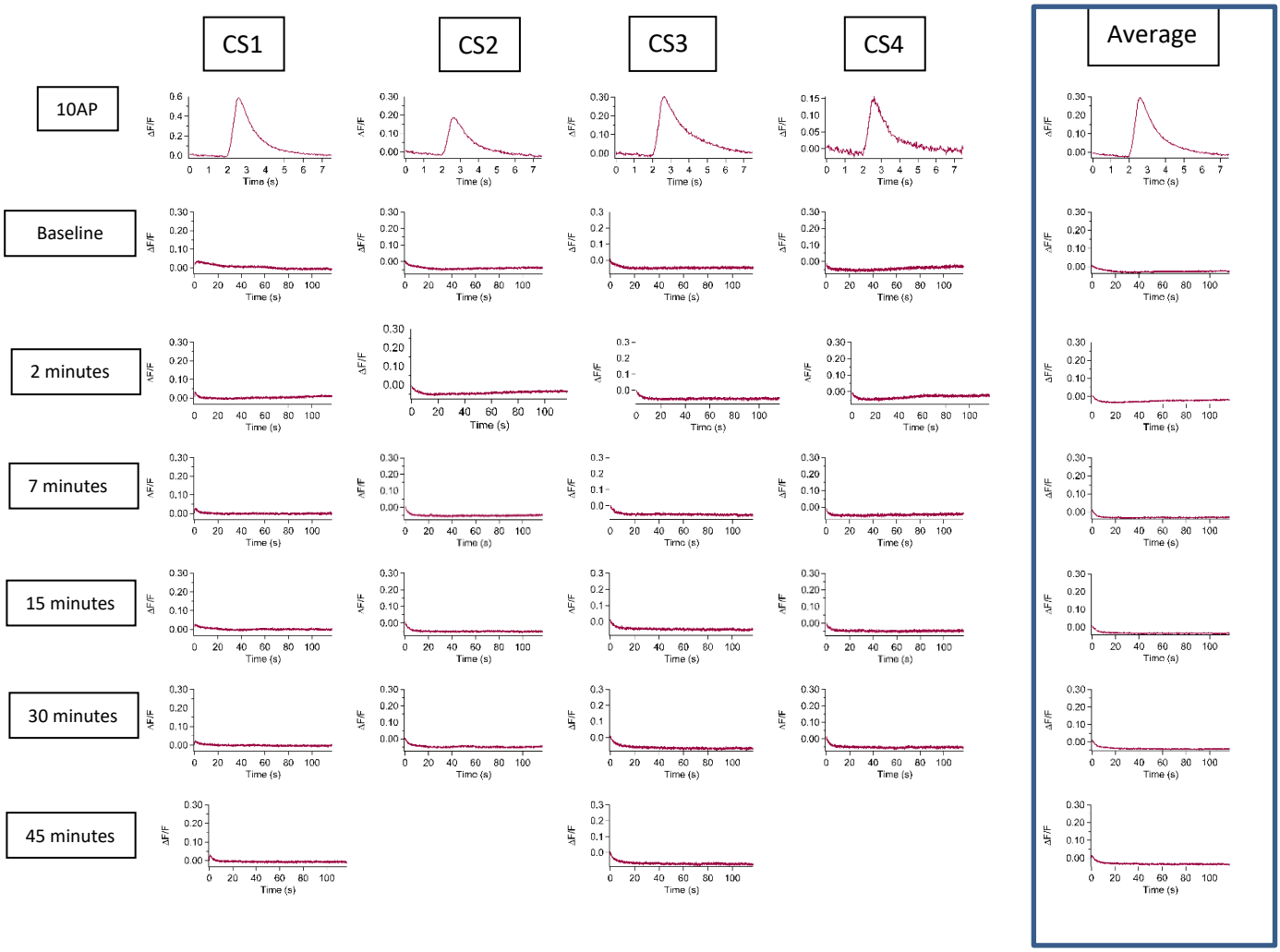


Figure 3.42- Overview of the effect of 10µM of Compound X on H2B-GCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3) and Coverslip 4 (CS4). The last column is the average of all coverslips for this condition (n=4). CS1 had 3 reacting cell bodies at DIV 20, CS2 showed 4 reacting cell bodies at DIV20 and CS3 has 1 reacting cell body at DIV20 and CS4 shows 1 cell body at DIV21. The total of cell bodies on this condition is 9.

Figure 3.44 shows the changes in spontaneous activity in CS1 and CS3 while CS2 does not show any responses to the compound. CS1 shows induction of spikes after the addiction of the compound X and at minute 15 the amplitude of the spikes increase severely. From minute 15 until minute 60, spikes become just one per 120s of recording. CS3 of Figure 3.44 shows an increase of firing rate and amplitude at minute 7, then, at minute 15, the frequency diminishes but in the last part of the recording there is a sudden boost of response. After that, at minute 30, there is again a sudden increase of amplitudes that at minute 45 stopped until the last seconds of the recording in which three spikes happen.

In **Figure 3.45**, the three coverslips show different responses to 1µM. CS1 show a radical increase and induction at of spikes at minute 7, then at minute 15 the spikes become less larger and frequent, to turn into a more constant firing mode at minute 45 and minute 60. Meanwhile, CS2 shows responses at minute 15 with induction of four spikes and then no more spikes like those happen until minute 60, just two small spikes at minute 45 and one sporadic one at minute 60. CS3 shows significant responses at minute 15 with small but constant spikes, and then at minute 30 the spikes become more frequent and with higher amplitude. After that, at minute 45, the firing rate decreases until minute 60.

Important to refer that there is no presentation of representative examples because all the cell bodies showed similar patterns with this compound, so the average of each coverslip represents the patterns on it.

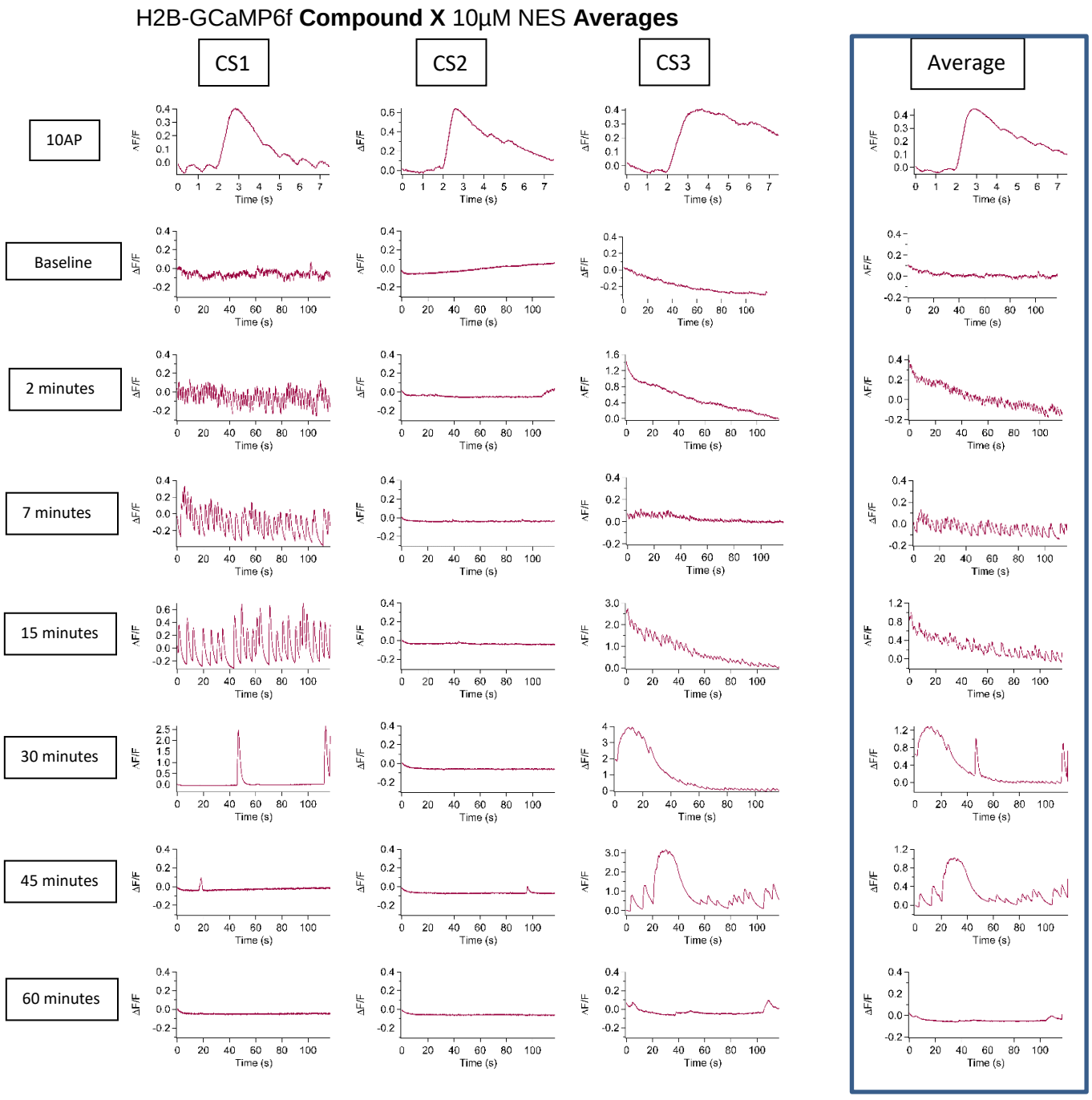


Figure 3.43- Overview of the effect of 10µM of Compound X on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2) and Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 6 reacting cell bodies at DIV 20, CS2 showed 5 reacting cell bodies at DIV21 and CS3 has 3 reacting cell body at DIV20. The total of cell bodies on this condition is 14.

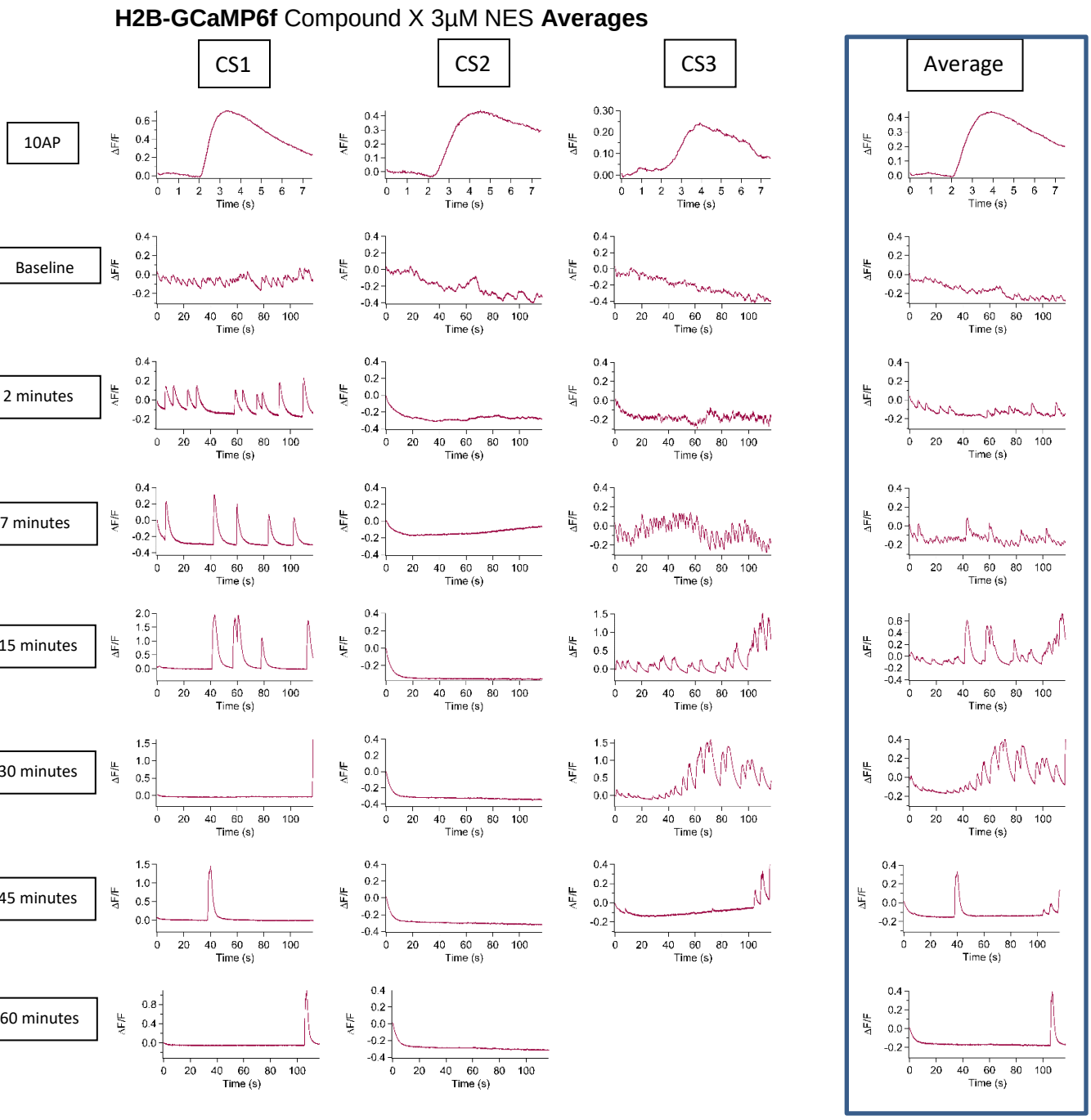


Figure 3.44- Overview of the effect of 3µM of Compound X on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2) and Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 3 reacting cell bodies at DIV 15, CS2 showed 1 reacting cell bodies at DIV22 and CS3 has 3 reacting cell body at DIV23. The total of cell bodies on this condition is 7.

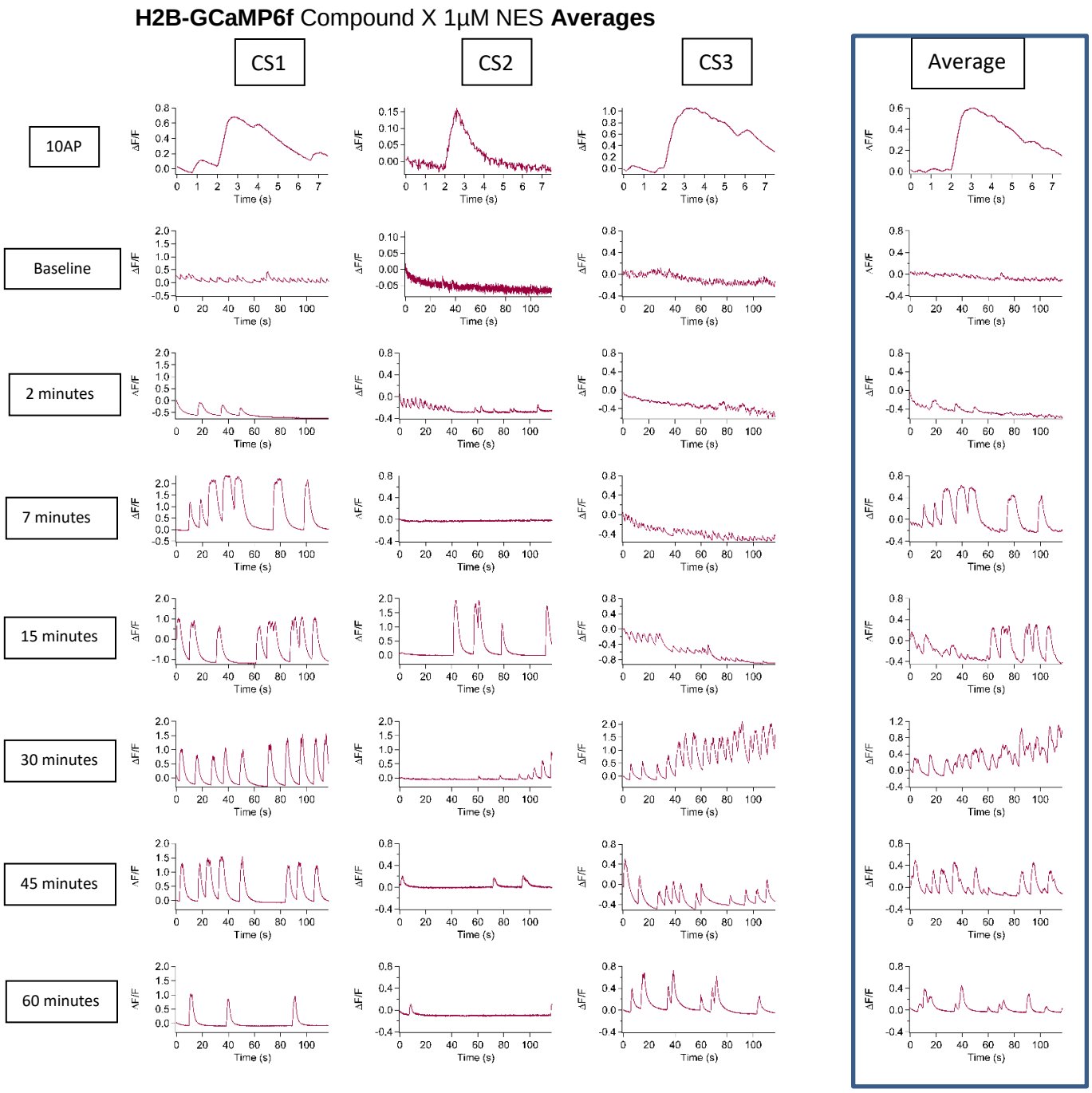
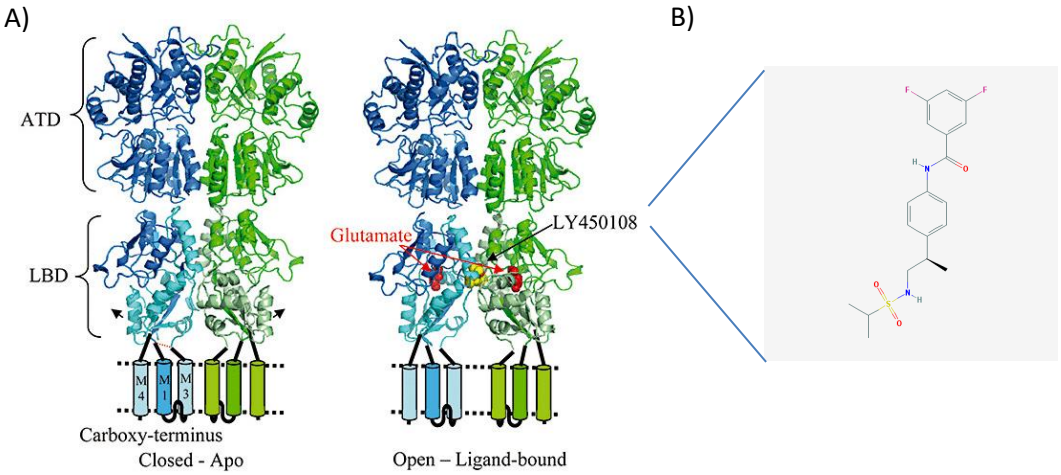


Figure 3.45- Overview of the effect of 1µM of Compound X on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2) and Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 5 reacting cell bodies at DIV 15, CS2 showed 1 reacting cell bodies at DIV23 and CS3 has 3 reacting cell body at DIV23. The total of cell bodies on this condition is 9.

LY450108 AMPA PAM

Considering that AMPA receptors are the synaptic receptors responsible not only for the fast-excitatory neurotransmission but also for the membrane depolarization that releases the magnesium- dependent block of NMDA receptors leading to their activation, AMPA receptors are an important target to neuronal excitability[10]. In addition, deficits in excitatory neurotransmission are one of the hypothesis behind death cell in Alzheimer's disease[10]. AMPA Positive Allosteric Modulators (PAM) are compounds that potentiate AMPA receptor activity by altering downstream signaling pathways. In addiction AMPA PAMs are involved in synaptic plasticity modulation and memory processes, showing therapeutically positive effects in the treatment of cognitive deficits and turning to be a possible candidate to use in the treatment of other CNS cognitive disorders such as depression and Parkinson disease [89]. LY450108 is one of the AMPA potentiators reported in the literature with possible use in cognitive disorders[10,89]. LY450108 is a biarylpropylsulfonamide identified by high-throughput screening technology against homomeric GluA4 [90]. LY450108's chemical structure is shown in figure 3.46.

AMPA receptor's conformational structure include two extracellular regions named amino-terminal domain (ATD) and ligand binding domain (LBD), in which the binding domain is responsible for agonists binding[90]. The binding site of LY450108 as well as many other AMPA PAM is at the interface between LBDs in which stabilizes the dimer where glutamate is bound and consequently decrease the level of the receptor's desensitization[90,91].



Adapted from Jhee et al., 2006

Figure 3.46- Conformational structure of a GluA2 receptor in the ligand-bound structure - open state – and Apo structure – close state. On the left, ligand binding domain (LBD) and amino-terminal domain (ATD) are identified on the apo structure as well as the movement of the domains on the glutamate binding. On the right, the structure of the open state of the receptor is shown including the binding of LY450108 (yellow) and glutamate (red). Two subunits are included in the picture, one in green and one in blue. B) Chemical structure of LY450108. Image from National Center for Biotechnology Information. PubChem Compound Database; CID=9843690, https://pubchem.ncbi.nlm.nih.gov/compound/9843690 (accessed June 12, 2017).

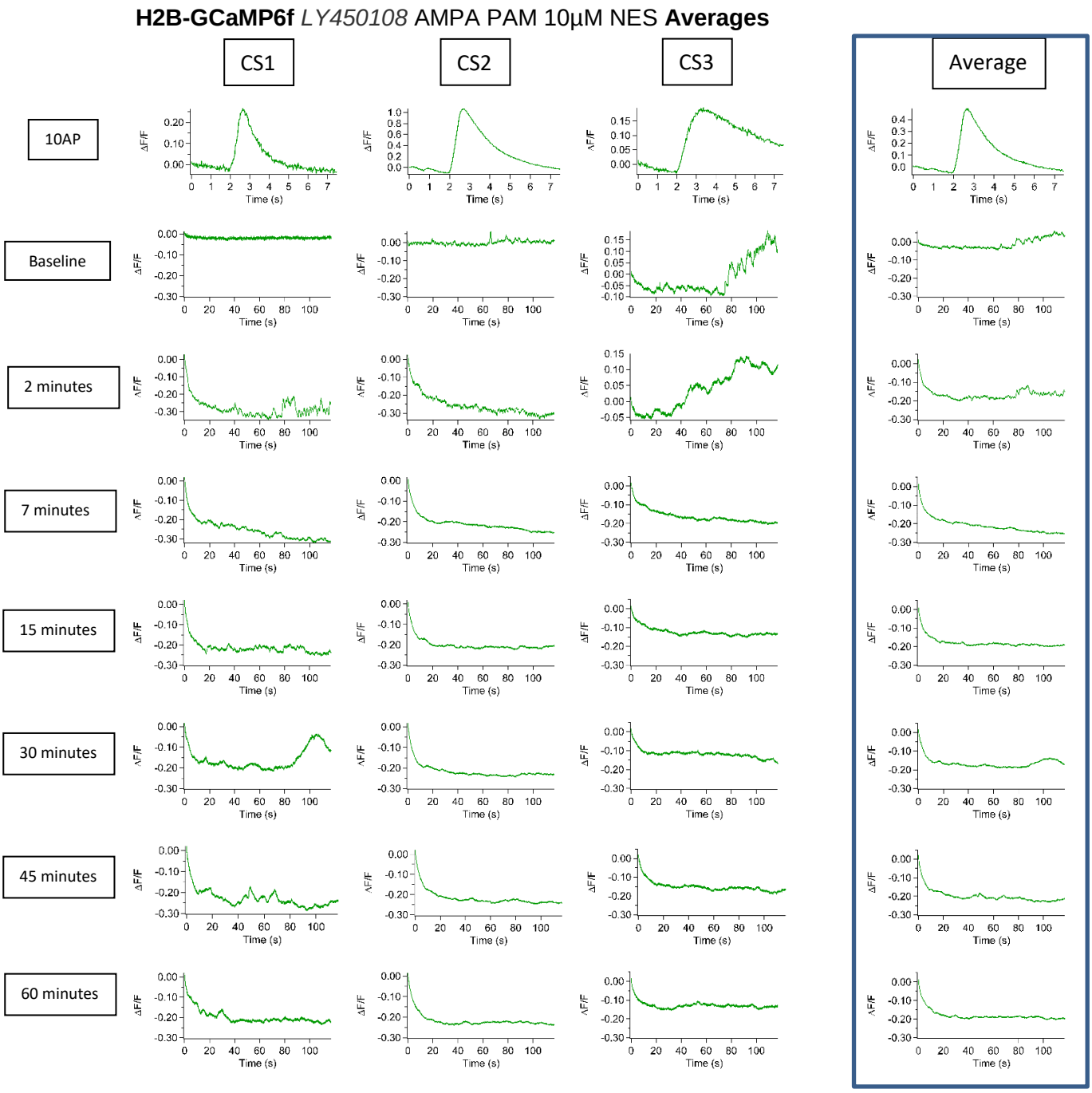


Figure 3.47- Overview of the effect of 10µM of AMPA PAM on H2B-GCaMP6f responses over time in the absence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 2 reacting cell bodies at DIV 21, CS2 showed 4 reacting cell bodies at DIV21 and CS3 has 2 reacting cell body at DIV21. The total of cell bodies on this condition is 8.

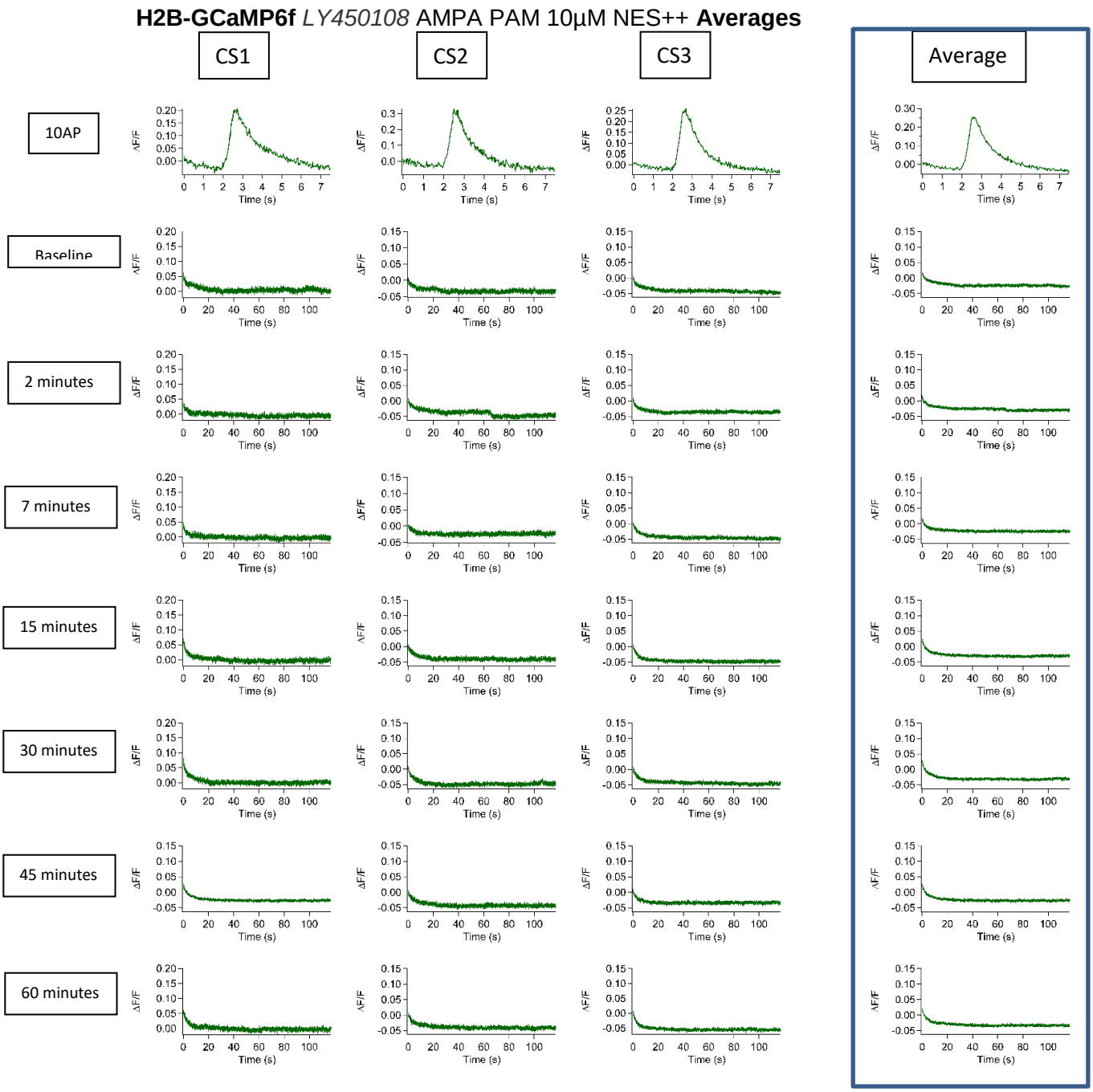


Figure 3.48- Overview of the effect of 10µM of AMPA PAM on H2B-GCaMP6f responses over time in the presence of synaptic blockers. The first three columns show the average of the responses over time per coverslip: Coverslip 1 (CS1), Coverslip 2 (CS2), Coverslip 3 (CS3). The last column is the average of all coverslips for this condition (n=3). CS1 had 2 reacting cell bodies at DIV 19, CS2 showed 4 reacting cell bodies at DIV20 and CS3 has 2 reacting cell body at DIV20. The total of cell bodies on this condition is 8.

Important to refer that there is no presentation of representative examples because all the cell bodies showed similar patterns with this compound, so the average of each coverslip represents the patterns on it.

Figure 3.47 shows small changes in the shape of the spontaneous activity after the addition of the compound, mostly on CS1, in which the profile of each time point shows different irregularities. CS2 and CS3 do not show significant changes over time, beside from minute 2 to minute 7 on CS3 where the peak decrease.
On **Figure 3.48** there are no visible changes with the addition of the compound.

Considering that all the compounds are dissolved in a final concentration of 0.1% DMSO, the same protocol was performed in the presence of synaptic blockers but instead of adding the compound to the cultures it was added only 0.1% DMSO. The average response to 0.1% DMSO in three different coverslips is shown in **figure 3.49**. 0.1% DMSO does not influence spontaneous activity in the presence of synaptic blockers, meaning that the responses observed in pharmacologic tests are exclusively due to the compound.

H2B-GCaMP6f 0.1% DMSO Control NES ++ **Average**

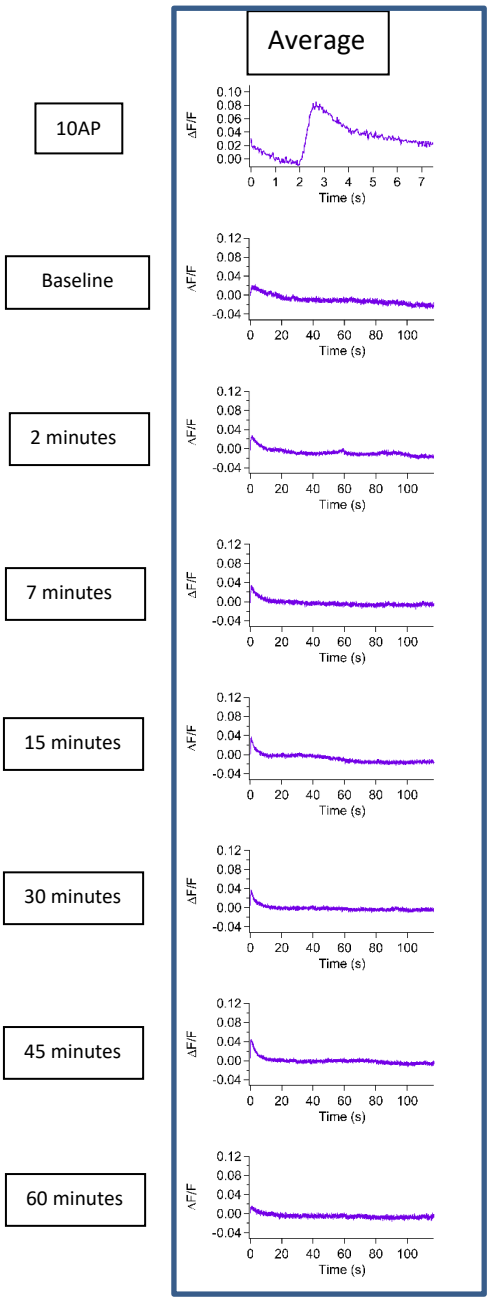


Figure 3.49- Overview of the effect of 0.1% DMSO on H2B-GCaMP6f responses over time in the presence of synaptic blockers. Average response of 3 different coverslips at DIV27. Each coverslip showed 2 reactive cell bodies. The total of cell bodies on this condition is 6.

4. Discussion

To a better understanding of the results, a table summarizing the characteristics of all the reporters used above was made (Table 4.1).

Table 4.1 – Resume of the reporters used throughout the project – SyjRCaMP1b, SyjRGECO1a, SyGCaMP6, Actin-GCaMP6f and H2B-GCaMP6f and respective target, fluorescent protein, light emitted by the reporter when excited, brief description or/and conclusion and references.

Reporter name	Target	Fluorescent Protein	Light emitted	Description/ Conclusion	References
SyjRCaMP1b	Synaptophysin (Pre-synaptic)	mRuby (RFP)	Red	Stable and ideal for optogenetics	Dana et al., (2016); Akerboom et al. (2013)
SyjRGECO1a	Synaptophysin (Pre-synaptic)	mApple (RFP)	Red	Fastest red GECI	Dana et al., (2016)
SyGCaMP6f	Synaptophysin (Pre-synaptic)	GFP	Green	Fastest and most sensitive green GECI	Chen et al. (2013)
Actin-GCaMP6f	Actin (Post-synaptic)	GFP	Green	Not conclusive	Mao et al. (2008)
H2B-GCaMP6	Histone (Nuclear)	GFP	Green	Well targeted and expressed in the nucleus	Dunn *et al. (2016)*

SyjRCaMP1b, SyGCaMP6f and SyjRGECO1a

SyjRCaMP1b and RCaMP1b

SyjRCaMP1b shows a good dynamic range yet its 1AP response was higher than the expected response, as described in the literature[23]. One possible explanation is that in Dana et al. (2016) jRCaMP1b was a cytoplasmic reporter while SyjRCaMP1b is a pre-synaptically targeted GECI, which can explain the increased amplitude for SyjRCaMP1b to 1 AP[27].

Nevertheless, SyjRCaMP1b shows stability of responses over one hour; however, it is important to note that it was tested only one hippocampal coverslip with a total of 113 synapses. SyjRCaMP1b stability can be explained by mRuby's known thermostability[29]. Furthermore, SyjRCaMP1b's amplitudes scale with the number of APs in response to electrical stimulation; more specifically in the range of 10 AP to 40AP where the increase is not so evident however it doesn't reach a saturation plateau. According to Dana et al. (2016), jRCaMP1b shows no saturation in the range of 1-160 APs[23]. The synaptophysin specific target of SyjRCaMP1b can increase the sensitivity of the reporter however with the increase of sensitivity comes a possible reduction of dynamic range and consequently the saturation plateau can be reached easier[27]. Regarding to the sensitivity of targeted GECIs according to Dreosti et al. (2009), it is visible that the amplitude in response of 1 AP is higher in SyGCaMP2 comparing to cytoplasmic GCaMP2, meaning that it shows more sensitivity when targeted pre-synaptically[27]. The increase in sensitivity on targeted

GECIs goes from 1 AP up to 10 AP, however the responses from 10 to 40 AP show no significant difference between targeted GECIs and cytoplasmic GECIs.[27]
Regarding kinetics, the constant of dissociation (K_{off}) of SyjRCaMP1b is 1.4388 ± 0.093431 seconds. Comparing to the literature, Dana et al. (2009) showed a half decay time of jRCaMP1b of 500 ± 45 ms[23] meaning that SyjRCaMP1b shows longer off kinetics response than cytoplasmic RCaMP1b. Further experiments are needed either to confirm or rule out this apparent discrepancy.

SyjRCaMP1b, SyjRGECO1a and SyGCaMP6f

Even though SyjRCaMP1b and SyjRGECO1a are both RFP-based fluorescent reporters, the sensitivity of SyjRCaMP1b's differs from the sensitivity of SyjRGECO1a[23]. It is known that mApple-based reporter's kinetics is faster than mRuby-based reporter kinetics: jRGECO1a show a half decay time of 390 ± 20 ms while jRCaMP1b shows a half decay time of 500 ± 45 ms[23]. These evidences can be visually confirmed in Figure 3.2A, in which SyjRGECO1a shows faster responses than SyjRCaMP1b.
Comparing to SyjRGECO1a, SyjRCaMP1b show smaller amplitude in responses up to 10 APs and shows less dynamic activity from 10AP to 40 AP yet still not reaching a saturation. It is known that RCaMP1b and jREGCO1a show a smaller dynamic range comparing to GCaMP6 indicators[23]. Comparing linearity of red shifted reporters to GCaMP6f, there is a significant difference (Figure 3.2B and Figure 3.16) between their behaviors, being SyGCaMP6f the most linear reporter of all the reporters in study.
GCaMP6f is considered the fastest cytoplasmic genetically-encoded calcium indicator in neurons and shows a dynamic range of 51.8±2.8%[22].SyGCaMP6f indeed shows the highest sensitivity comparing to SyjRCaMP1b, SyjRGECO1a and H2B-GCaMP6f as well as show a dynamic range higher than 51.8±2.8%..

Actin-GCaMP6f

The fact that actin-GCaMP6f showed expression along the neurons instead of showing punctuated and preferentially co-localized expression with PSD-mKate2, means that Actin-GCaMP6f is not well targeted to post synapses. Although there is some punctuated expression in the second row (Figure 3.4), they do not colocalize well with PSD-mKate2. According to Mao et al. (2008) this construct was difficult to localize in spines due to dimness of the signal. Since actin was not precisely localized to post synapses, no further characterization was performed in this reporter. Even though two biological replicates were tested, actin expression along the neuron could be for instance due to the amount of DNA used. Further studies should be performed to clarify these results.

H2B-GCaMP6f

Knowing that 1.panneuronal probes expression in zebrafish larvae has been shown to be more active up to 7 days post fertilization making the imaging of larvae mature stages a more challenging task and 2. GECIs as GCaMP3 are excluded from the nucleus[92] which limits the resolution of functional fluorescence responses during imaging; the development of a reporter well targeted to the nucleus which can be expressed in later stages of neurons maturation can overcome these drawbacks[93]. The design and creation of AAV:H2B-GCaMP6f construct can reliably report an AP in primary cultured neurons and can facilitate calcium imaging by improving the resolution of the signals as well as the wide expression

of a GECI into the development and maturation stages of not only zebrafish but also primary neuronal cultures.

Expression

Comparing not only the results of Kim et al. (2014) regarding the expression in dissociated rat hippocampal neurons of a transfected GCaMP3 linked to a nuclear sequence, but also the results of the expression of H2B-GCaMP6f in transgenic zebrafish in Dunn et al.(2016), our AAV:H2B-GCaMP6f construct shows a similar expression and shape. The high basal fluorescence of H2B-GCaMP6f makes possible the immediate location of cell nucleus at rest, which is an advantage when performing one single cell or/and network live imaging. On the other hand, having a high basal fluorescence can lead to saturation of the signal more easily. The high basal fluorescence might be due to the free diffusion of calcium from the nuclear cytoplasm to the nucleus[94] and due to a slower extrusion of calcium from the nucleus[95]. The different levels of expression of calcium buffers and pumps in the inner nuclear membrane can lead to slower extrusion of calcium in the nucleus when compared to the cytoplasm. The lower level of calcium buffers and pump in the nucleus is one of the mechanism behind of the slower decay time of response in nuclear responses comparing to synaptic responses, as it is visible on the comparison graph between H2B-GCaMP6f, SyGCaMP6f and SyjRGECO1a in figure 3.16.

Regarding H2B-GCaMP6f expression, with MOI 100, the reporter showed a good expression as well as a good targeting to the nucleus. The perfect co-localization of H2B-GCaMP6f with NeuN marker on the immunostainings of both biological replicates confirmed that H2B-GCaMP6f is strictly expressed only in the nucleus of the neurons.

Dynamic range and sensitivity

H2B-GCaMP6f exhibited a linear response when stimulated with train of different number of APs, especially above 10AP.

In the presence of synaptic blockers, the nucleus only receives the calcium inputs coming from external electrical stimulation evoked depolarization, so the response scales linearly with the number of APs. With the blockage of NMDA and AMPA receptors, generation of action potential rely on the voltage-dependent channels, meaning that only basal network activity is being measured[43]. In the absence of synaptic blockers, it was measured the normal activity of the network in which glutamatergic activation of NMDA and AMPA receptor also influences the nuclear calcium transients[45]. Considering that in the absence of synaptic blockers, nuclear responses are a summation of synaptic inputs and external stimulation, this can explain why H2B-GCaMP6f calcium signals are broader and take longer to reach the basal line as well as it can explain the difference in the amplitude of 1AP response in the presence and absence of synaptic blockers in Figure 3.12.

It is important to compare H2B-GCaMP6f performance to other reporters. Regarding 1AP response of H2B-GCaMP6f, SyGCaMP6f and SyjRGECO1a, both pre-synaptic reporters – SyGCaMP6f and SyjRGECO1a- showed higher amplitude than H2B-GCaMP6f. There is no significant difference in amplitudes of SyGCaMP6f and SyjRGECO1a.There are several factors that can influence pre-synaptic signals such as the extrusion and buffering of calcium by membrane calcium exchangers, pumps and internal calcium stores[95]. Also, the geometry and size of the pre synaptic compartment as well as the density of voltage dependent calcium channels[27].

Comparing nuclear and synaptic signals

It was performed multiplex imaging to compare nuclear and synaptic responses simultaneously in the same region. H2B-GCaMP6f was expressed strictly in the nucleus while SyjRGECO1a was pre-synaptically targeted. Importantly, it was not compared nuclear and synaptic responses from the same neuron but from a same region containing between 3 to 7 nuclei expressing H2B-GCaMP6f.

It is visible that synaptic responses occur before nuclear ones. The delay of nuclear responses can be related to the limited number of nuclear pores comparing to the number of synaptic calcium channels[95]. Multiplex imaging recordings confirmed the delay of the nuclear responses comparing to synaptic responses (data shown in digital version).

Regarding the comparison between H2B-GCaMP6f and SyGCaMP6f responses, they both show linear responses as it is described in literature[22], however SyGCaMP6f shows more linearity than H2B-GCaMP6f with trains of APs up to 10AP. From 10AP to 40AP H2B-GCaMMP6f and SyGCaMP6f show similar performance, although SyGCaMP6f exhibits constantly higher amplitudes as explained above.

Pharmacological characterization of H2B-GCaMP6f

A table with a resume regarding the pharmacologic results in the presence of synaptic blockers is shown below (Table 4.2). Results regarding the absence of synaptic blockers were not shown in the table since the responses are not constant and by visual analysis it becomes hard to get some patterns. Further software analysis must be done.

Knowing that AMPA PAM would potentiate AMPA receptor function, the pharmacological results in the presence of synaptic blockers confirm that this compound acts on synaptic receptors because there was no change in spontaneous activity when an AMPA PAM was added in cultures - with AMPA receptors previously blocked by NBQX. Nevertheless, in the absence of synaptic blockers there were slight changes over time. AMPA is the synaptic receptor responsible for fast excitatory transmission and membrane depolarization[10]. Even though the changes are smaller and not constant, it is evident that the compound altered neuronal excitability activity via modulation of synaptic activity.

Knowing that **LY450108 (AMPA PAM)** decrease the level of the receptor's desensitization could be an explanation for the increase of the firing pattern in CS1. Although in CS2 there is no significant responses to the compound and in the CS3 it looks like the compound reduce the initial neuronal excitability. There is no variability between cell bodies with this compound.

With **compound X**, intrinsic neuronal excitability was not altered when added 10 µM to the cultures in NES++, which raise the question regarded to the mechanisms of action - does this compound act on synaptic activity, as LY450108? At 10µM, the changes seem like a synaptic adaption, in which the short-term reaction is an acceleration of the firing rate, which could be a neuronal compensation mechanism to an external stimulus or a mechanism towards neuronal death. This preliminary pharmacological test can indicate toxic effects on this concentration, although further tests need to be done. By adding 3 µM, the firing rate is faster and constant but also show the same pattern of 10 µM responses after 45 minutes. Addition of 1 µM shows significant changes in synaptic plasticity, where three coverslips show amplitudes higher than 10 AP amplitude as well as more constant spikes over one hour. 1µM seems to show a more normal and constant neuronal response although no conclusions can be taken without further studies. One drawback of this experiments is the control of synaptic activity over one hour without compound. It was

performed a control in the presence of synaptic blockers to study the influence of 0.1% DMSO on the cultures yet no control in the absence of synaptic blockers was performed.
Considering that potassium is involved in hyperpolarization and resting membrane potential phase on AP cycles, voltage-dependent potassium channels are known to play a role in the creation and propagation of APs [85].
Knowing that **KV1.3 blocker** is a compound that blocks KV1.3 voltage dependent potassium channels, it is expected that the presence of this compound influence intrinsic neuronal excitability through alteration of the firing threshold[9].
There was one cell body in eleven reacting cell bodies that reacts to the addition of this compound and started to fire. The firing threshold can vary in neurons because it is dependent on axonal diameter, density of voltage dependent sodium channels and properties of the sodium channel [96]. One hypothesis is that the blockade of potassium channels turns the threshold of firing less negative due to less influx of potassium to intracellular space. In the absence of synaptic blockers, it also increases the firing rate of the neurons originating different patterns of firing.
As explained above intrinsic neuronal properties which include resting membrane potential, differ from neuron to neuron. H2B-GCaMP6f sensitivity to monitor small calcium transients is lower than SyGCaMP6f sensitivity so smaller changes induced by 10 µM are preferably detectable by SyGCaMP6f, although one cell body could have shown significantly bigger responses which were detectable by the nuclear reporter.
Adding 3 µM does not change intrinsic membrane properties, so one hypothesis is the concentration not being enough to induce intrinsic changes, as well as 1 µM also does not show any significant changes. Meanwhile, in the absence of synaptic blockers, there were significant changes mainly in the amplitude of the spikes instead of firing rate.
Regarding addition of 1 µM of KV1.3blocker in NES, one coverslip didn't show any response, other coverslip has as average the responses of one cell body that was in stress and towards cell death. CS3 showed an extremely high spontaneous activity without compound, and then a decrease of that abnormal amplitude over time.CS1 was the one with more normal reactivity although no significant changes was shown at this concentration.
Comparing H2B-GCaMP6f with SyGCaMP6f responses in the presence of synaptic blockers, the same concentration of 10 µM showed more changes in synaptic calcium signaling than in nuclear calcium signaling, explained above the differences between nuclear responses and synaptic ones. SyGCaMP6f experiments confirm that 10 µM indeed alter intrinsic membrane properties although H2B-GCaMP6f is less sensitive to smaller changes than SyGCaMP6f. Adding 3 or 1 µM did not lead to any changes in the firing rate of the spontaneous activity. In addition, the difference between patterns in synapses side by side is clear and significant (with SyGCaMP6f, in NES++) however it is known that synapses can vary on their responses in normal network activity [27]. In NES, at 10 µM the firing rate shows different patterns over time yet the fire rate is mostly constant over time, raising a hypothesis of a mechanism of neuronal adaptability. At 3 µM the differences between coverslips are not as clear as at 10 µM NES but still significant. Surprisingly by adding a lower concentration (1 µM), one coverslip reacts by firing high and broad spikes and then increased drastically the firing rate, on the contrary CS2 and CS3 increased first firing rate and decreased over time. Representative examples at 1 µM and 3 µM show mainly differences in amplitude, although in CS1 representative examples it is clear two different patterns over time.

This variability between coverslips and cell bodies responses can due to the heterogeneity of the cultures, here we cannot specify the type of neurons responding in each recording, which could be inhibitory or excitatory neurons, neither the type of inputs they are influenced by. Astrocytes are also present and can play a role on the inputs that neurons received.

Table 4.2 – Overview of the results regarding intrinsic neuronal excitability through pharmacologic tests, using H2B-GCaMP6f and SyGCaMP6f. Overview of all the experiments in the presence of synaptic blockers, using H2B-GCaMP6f and SyGCaMP6f. Colors on the table according to the color of the respective graphs. Relative intensity of the firing represented by "+". The more the firing rate, the more "+" showed in the table. The firing rate does not take into consideration the amplitude and it is an overview of the firing by visual analysis. No software analysis was taken.

	H2B-GCaMP6f					SyGCaMP6f		
	KV 1.3 blocker			Compound X	AMPA PAM	KV 1.3 blocker		
	10µM	3µM	1µM	10µM	10µM	10µM	3µM	1µM
Baseline	-	-	-	-	-	-	-	-
2 minutes	-	-	-	-	-	+++	-	-
7 minutes	+	-	-	-	-	+++	-	-
15 minutes	++	-	-	-	-	+++	-	-
30 minutes	-	-	-	-	-	-	-	-
45 minutes	-	-	-	-	-	-	-	-
60 minutes	-	-	-	-	-	-	-	-

5. Conclusion

The characterization of a reporter is based on its dynamic range, linearity, kinetics and stability over time. We fully characterized SyjRCaMP1b and H2B-GCaMP6f and compare with SyGCaMP6f and SyjRGECO1a. As it was expected, SyjRCaMP1b is less sensitive than SyGCaM6f and SyjREGO1a, although it showed enough sensitivity, stability over time and dynamic range to be used as a pre-synaptic reporter in synaptic function assays. Its characterization is important since it is a good reporter to combine with further optogenetics experiments or to use in multiplex imaging. Additionally, the post-synaptic reporter Actin-GCaMP6f was tested, however further experiments need to be performed to characterize this reporter.

We also studied, by multiplex imaging, the differences in calcium signaling between synapses and the nucleus, using H2B-GCaMP1b as nuclear reporter and SyjRGECO1a as a pre-synaptic reporter. Synaptic calcium responses to trains of different number of action potentials at 20Hz were clearly faster and higher than nuclear responses.

We have developed a new construct (H2B-GCaMP6f) to measure neuronal excitability by monitoring nuclear calcium signaling. This nuclear reporter is well targeted and expressed in the nucleus exhibiting more linear responses to train of different action potentials at 20Hz comparing to synaptic reporters. In addition, this nuclear reporter can be used with spectrally distinct reporters to monitor distinct synaptic events, such as synaptic and nuclear responses simultaneously. Moreover, H2B-GCaMP6f is suitable for preliminary pharmacological tests and it can be used for further phenotypic or target-based drug screening efforts in the context of Alzheimer’s Disease or other neurological diseases.

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7. Appendix

Methods

Molecular Biology – AAV and plasmid sequences

It is available the complete sequence as well as it is highlighted the sequence related to the reporter.
As a promoter, a human synapsin (HYSN) promoter was integrated in all the viral constructs. For the plasmid, a human cytomegalovirus (CMV) enhancer and promoter were integrated in the virus.

H2B-GCaMP6f

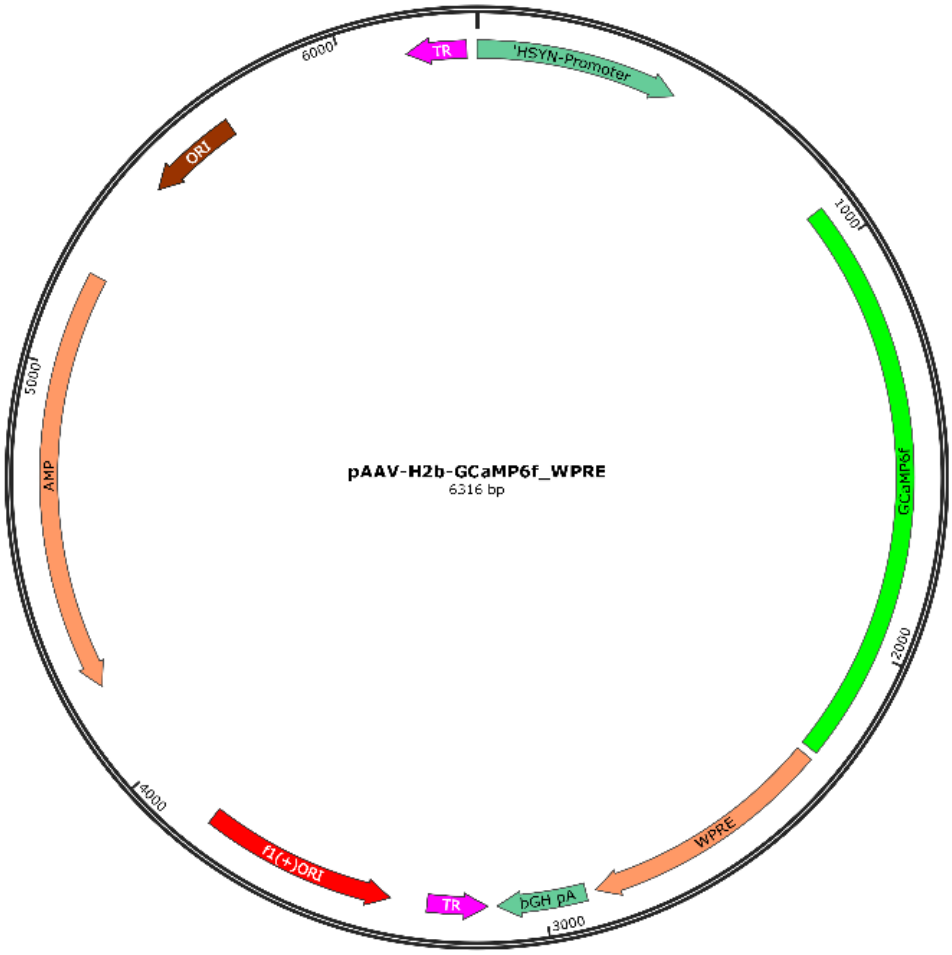

Figure 7.1- Circular map of AAV-6-SH2B-GCaMP6f_WPRE (I) construct.

H2B-GCaMP6f sequence

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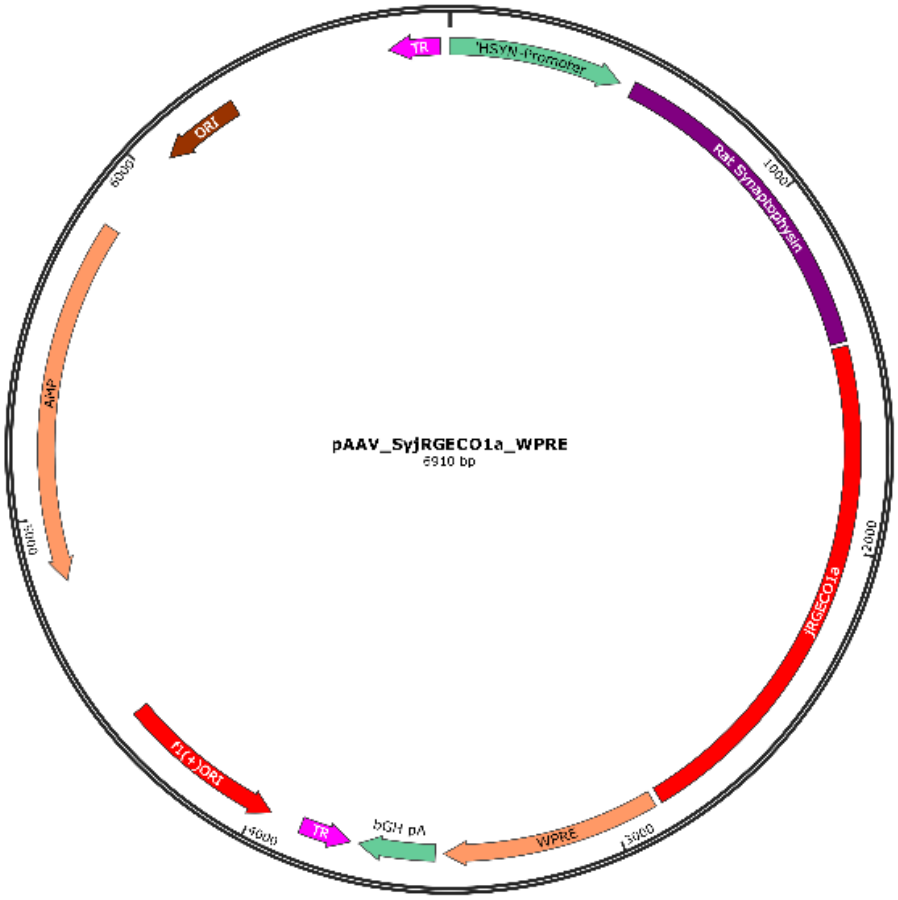

Figure 7.2 - Circular map of AAV-6-SyjRGECO1a_WPRE (I) construct.

SyjRGECO1a sequence

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APPENDIX - 114

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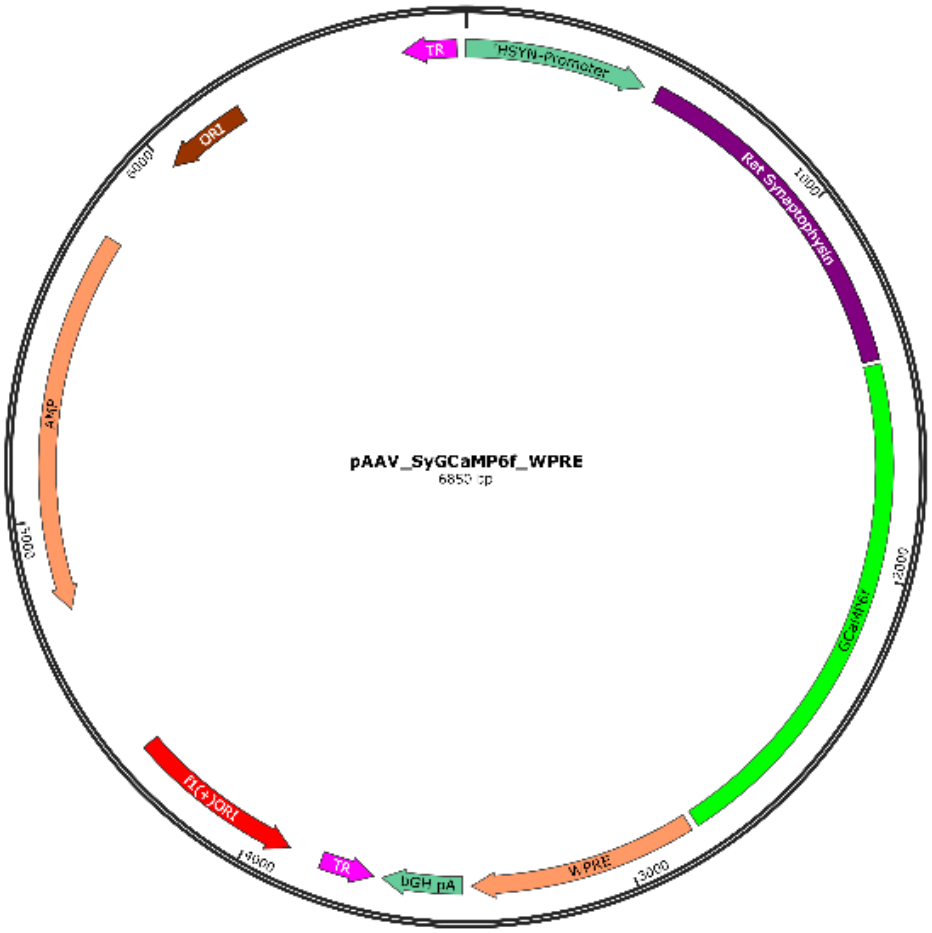

Figure 7.3 - Circular map of AAV-6-SyGCaMP6f (I) construct.

SyGCaMP6f sequence

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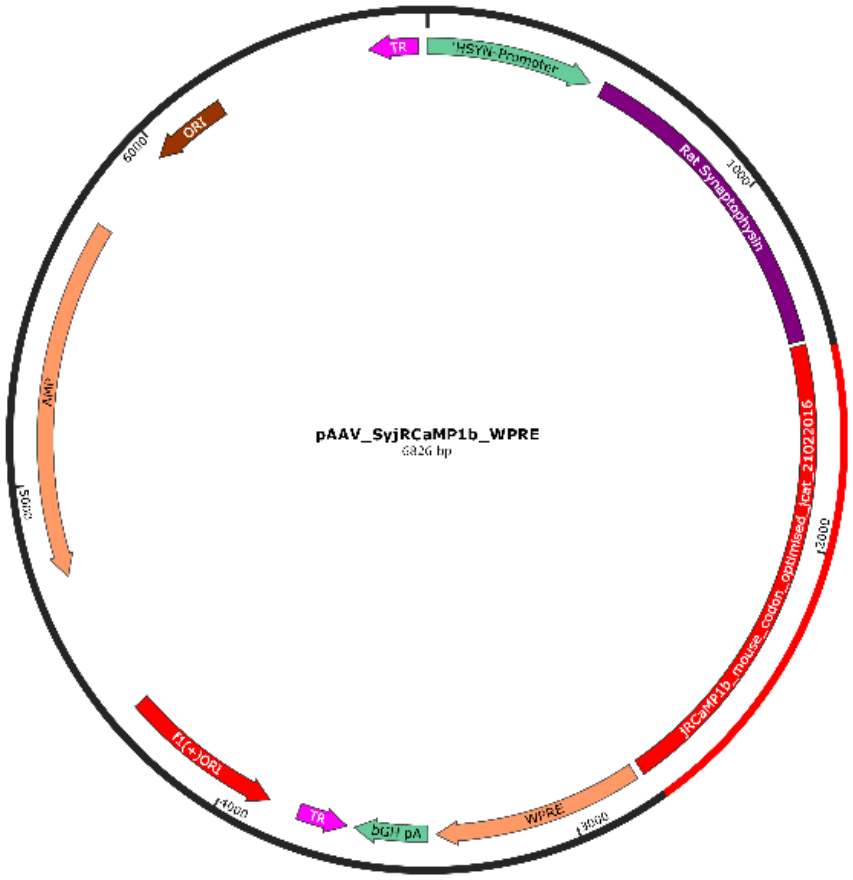

Figure 7.4 - Circular map of AAV-6-SyjRCaMP1b_WPRE (I) construct.

SyjRCaMP1b sequence

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APPENDIX - 120

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Actin-GCaMP6f plasmid

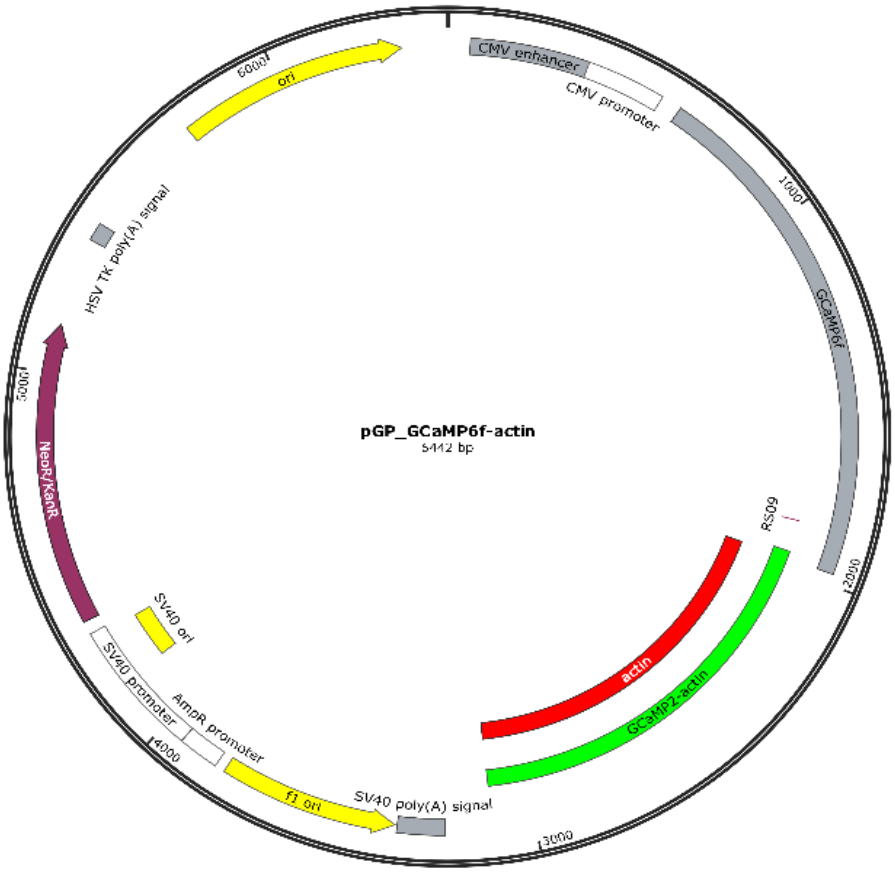

Figure 7.5 - Circular map of pGP_GCaMP6f-actin construct.

Actin-GCaMP6f sequence

tagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgc
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CTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTA
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CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG
GGCGGTACCGGAGGGAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGT

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GCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGTGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCAC
CGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCA
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CCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGG
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CGGACCAACTGACTGAAGAGCAGATCGCAGAATTTAAAGAGGAATTCTCCCTATTTGAC
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gtattaccgccatgcat

SCRIPT

FUNCTIONS

```
#pragma rtGlobals=3     // Use modern global access method and strict wave access.
#include <Waves Average>

Function makeGraphs()
   string selectedWavesFolder = "Selected Waves"

   //Create empty list to which we are adding the waves
   variable x
   String apfolder = GetDataFolder(0)
   setdatafolder root:$(apfolder):$(selectedWavesFolder)
   string selectedwaves = WaveList("*",";", "DIMS:1")
   Display $(stringfromlist(0, selectedwaves))
   variable i
   for(i=1; i<itemsinlist(selectedwaves); i+=1)
      AppendToGraph $(stringfromlist(i, selectedwaves))
   endfor
   setdatafolder root:$(apfolder)
   plotAvrgWave("appendwave")
End

Function createDataFiles()
   Variable refNum = 0
   Open /P=home refNum as "amplitudeAverage.txt"
   fprintf refNum, "Capture\t ActionPotentials\t Amplitude\r"
   Close /A
   //create txt to hold area under curve data
   Open /P=home refNum as "areaundercurve.txt"
   fprintf refNum, "Capture\t ActionPotentials\t AreaUnderCurve\r"
   //create txt to hold kinetics data
   Close /A
End

Function createDataFolders()
   String selectedWavesFolder = "Selected Waves"
   string excludedWavesFolder = "Excluded Waves"
   string dfwavesfolder = "DFswaves"
   String averagefolder = "Average wave"
   NewDataFolder $selectedWavesFolder
   NewDataFolder $excludedWavesFolder
   NewDataFolder $dfwavesfolder
   NewDataFolder $averagefolder

   getVideoName()
   getapfoldername()
```

```
End

Function getVideoName()
    string dfWaveList=Wavelist("*DF",";","DIMS:2")
    String apfolder_n = stringfromlist(0,dfWaveList)
    String /G dfwavename = apfolder_n
    String removeZero = RemoveEnding(apfolder_n, "_QA_DF")
    String /G videoName = removeZero
    Print videoname
End

Function getAPfoldername()
    String /G apfolder = getdatafolder(0)
End

Function getDFwaves()
    variable i
    String /G apfolder, dfwavename
    Wave dfwave = $dfwavename

    //get size of df wave
    variable numberDFwaves = dimsize(dfwave, 1)
    variable lengthwave = dimsize(dfwave, 0)
    setdatafolder root:$(apfolder):dfswaves
    for(i=0; i < numberDFwaves; i+=1)

        Make /O/N=(lengthwave) newwave=p
        newwave[]=dfwave[p][i]
        String newname = dfwavename + num2str(i)
        Rename newwave, $newname
    endfor
    setdatafolder root:$(apfolder)
End


//automatic selection of waves based on 2xSD of baseline and amplitude
Function dfWaveSelection()
    //set current folder to dfwavesfolder
    String selecteddffolder = "Selected Waves"
    String excludeddffolder = "Excluded Waves"
    String /G apfolder
    setdatafolder root:$(apfolder):dfswaves

    //start variables for loop
    DFREF dfr=root:$(apfolder):dfswaves
    Variable numberDFwaves = CountObjectsDFR(dfr, 1)
    variable i

    //calculate upper and lower confidence limits for baseline and amplitude stats
```

```
    variable basupplim = avrgbaseline(apfolder) + (1.96*stdbaseline(apfolder))
    variable baslowlim = avrgbaseline(apfolder) - (1.96*stdbaseline(apfolder))
    Variable ampupplim = avrgamplitude(apfolder) + (1.96*stdamplitude(apfolder))
    Variable amplowlim = avrgamplitude(apfolder) - (1.96*stdamplitude(apfolder))

//  calculates amplitude and baseline for each individual DFwave and evaluates if it is within 2xSD for one or another parameter
    for(i=0; i < numberDFwaves; i += 1)
        String singleDFwave_name = GetIndexedObjNameDFR(dfr, 1, i)
        Wave singleDFwave = $singleDFwave_name
        Variable dfwaveamplitude = singleDFamplitude(singleDFwave_name)
        Variable dfwavebaseline = singleDFbaseline(singleDFwave_name)

        if((dfwavebaseline < baslowlim) | (dfwavebaseline > basupplim) |(dfwaveamplitude < amplowlim) | (dfwaveamplitude > ampupplim))
            Duplicate /O singleDFwave, root:$(apfolder):$(excludeddffolder):$(singleDFwave_name)
        else
            Duplicate /O singleDFwave, root:$(apfolder):$(selecteddffolder):$(singleDFwave_name)
        endif
    endfor
    setdatafolder root:$(apfolder)
End
```

AVERAGE WAVE

```
#pragma rtGlobals=3     // Use modern global access method and strict wave access.
#include <Waves Average>

Function singleAverageWave()
    //create the average wave
    string selectedWavesFolder = "Selected Waves"
    String averagewavefolder = "Average wave"
    String /G apfolder, dfwavename, videoname
    String avrgWaveName = videoname + "_average"
    setdatafolder root:$(apfolder):$(selectedwavesfolder)
    fWaveAverage(WaveList("*", ";","DIMS:1"), "", 0,1 , avrgWaveName, "")
    Wave avrgWave = $avrgWaveName
    MoveWave avrgWave, root:$(apfolder):$(averagewavefolder):$(avrgWaveName)
    setdatafolder root:$(apfolder)
End

Function plotAvrgWave(type)
    String type
```

```
    String /G apfolder
    String averagewavefolder = "Average wave"
    SetDataFolder root:$(apfolder):$(averagewavefolder)
    String avrgwavelist = WaveList("*average",";","DIMS:1")
    String avrgwave_name = StringFromList(0, avrgwavelist)
    Wave avrgwave =  $avrgwave_name
    Print avrgwave_name
    //Display avrgwave
    If(stringmatch(type,"appendwave") == 1)
        AppendToGraph avrgwave
        String avrgTrace = NameOfWave(avrgwave)
        ModifyGraph lsize($avrgTrace)=5,rgb($avrgTrace)=(0,0,0)
    elseif(stringmatch(type,"displayonly") == 1)
        Display avrgwave
    endif
    setdatafolder root:$(apfolder)
End

//Function that calculates the average from all the waves of one video for a single action potential
Function averageWaves()
    //List of all waves to average
    String waves4average = WaveList("*", ";","WIN:Graph0")
    Print waves4average
End

Function averageAllwaves()
    String /G apfolder
    DFREF dfr = root:$(apfolder):
    variable numberFolders = CountObjectsDFR(dfr, 4)
    variable apNumbers, i
    for(i=1; i<numberFolders; i+=1)
        string folderName = GetIndexedObjNameDFR(dfr, 4, i)
        setDataFolder root:$folderName
        averageWaves()
        restoreCurrentFolder()
    endfor
End
```

AMPLITUDE

```
#pragma rtGlobals=3     // Use modern global access method and strict wave access.
#include "Tools window"

Function singleDFamplitude(singleDFwave_name)
   String singleDFwave_name
   Wave singleDFwave = $singleDFwave_name
   variable amplitude = WaveMax(singleDFwave)-faverage(singleDFwave,0.15,1.74)
   return amplitude
End

Function multipleDFamplitude()
   variable x
   String selecteddffolder = "Selected Waves"
   String nameAPs = "AP40"
   String nameAmplitudeFile = "amplitude_DFs.txt"
   setdatafolder root:$(nameAPs):$(selecteddffolder)
   string dfWaveList=Wavelist("*",";","DIMS:1")
   Print dfwavelist
   DFREF dfr = root:$(nameAPs):$(selecteddffolder)
   variable numberofWaves = CountObjectsDFR(dfr, 1)

   for(x=0; x < numberofWaves; x+=1)
      String videoNameSin_name = GetIndexedObjNameDFR(dfr, 1, x)
      //Wave videoNameSin = $videoNameSin_name
      String amplitudeX = num2str(singleDFamplitude(videoNameSin_name))
      Print amplitudeX
   endFor
End

Function allamplitudeDFs()
   DFREF dfr = root:
   variable numberFolders = CountObjectsDFR(dfr, 4)
   print numberFolders
   variable apNumbers, i
   for(i=0; i<numberFolders; i+=1)
      string folderName = GetIndexedObjNameDFR(dfr, 4, i)
      if(StringMatch(folderName, "AP*")==1)
         setDataFolder root:$folderName
         print folderName
         multipleDFamplitude()
         restoreCurrentFolder()
      endif
   endfor
   Close /A
End
```

```
Function singleDFbaseline(singleDFwave_name)
    String singleDFwave_name
    Wave singleDFwave = $singleDFwave_name
    Variable baselineavrg = faverage(singleDFwave,0.15,1.74)
    return baselineavrg
End

//calculates average of all the amplitudes from dfs extrated from a single DF multidimentional wave
Function avrgamplitude(apfolder)
    String apfolder
    DFREF dfr=root:$(apfolder):dfswaves
    Variable numberDFwaves = CountObjectsDFR(dfr, 1), i, amplitudesum = 0

    for(i=0; i < numberDFwaves; i += 1)
        String singleDFwave_name = GetIndexedObjNameDFR(dfr, 1, i)
        Variable dfwaveamplitude = singleDFamplitude(singleDFwave_name)
        amplitudesum += dfwaveamplitude
    Endfor
    Variable amplitudeavrg = amplitudesum / numberDFwaveS
    Return amplitudeavrg

End

//calculates average of all the baselines from dfs extrated from a single DF multidimentional wave
Function avrgbaseline(apfolder)
    String apfolder
    DFREF dfr=root:$(apfolder):dfswaves
    Variable numberDFwaves = CountObjectsDFR(dfr, 1), i, baselinesum = 0

    for(i=0; i < numberDFwaves; i += 1)
        String singleDFwave_name = GetIndexedObjNameDFR(dfr, 1, i)
        Variable dfwavebaseline = singleDFbaseline(singleDFwave_name)
        baselinesum += singleDFbaseline(singleDFwave_name)
    Endfor

    Variable baselineavrg = baselinesum / numberDFwaves
    Return baselineavrg
End

//calculates standard deviation of all the baselines from dfs extrated from a single DF multidimentional
wave
Function stdbaseline(apfolder)
    String apfolder
    DFREF dfr=root:$(apfolder):dfswaves
    Variable numberDFwaves = CountObjectsDFR(dfr, 1), i, summeandiffbas = 0

    for(i=0; i < numberDFwaves; i += 1)
        String singleDFwave_name1 = GetIndexedObjNameDFR(dfr, 1, i)
```

```
      Variable meandiffbas = (singleDFbaseline(singleDFwave_name1) - avrgbaseline(apfolder))*(singleDFbaseline(singleDFwave_name1) - avrgbaseline(apfolder))
      summeandiffbas += meandiffbas
   endfor

   Variable baselinestd = sqrt((1/(numberDFwaves-1)) * summeandiffbas)
   return baselinestd
End


//calculates standard deviation of all the averages from dfs extrated from a single DF multidimentional wave
Function stdamplitude(apfolder)
   String apfolder
   DFREF dfr=root:$(apfolder):dfswaves
   Variable numberDFwaves = CountObjectsDFR(dfr, 1),  i, summeandiffamp = 0

   for(i=0; i < numberDFwaves; i += 1)
      String singleDFwave_name1 = GetIndexedObjNameDFR(dfr, 1, i)
      Variable meandiffamp = (singleDFamplitude(singleDFwave_name1) - avrgamplitude(apfolder))*(singleDFamplitude(singleDFwave_name1) - avrgamplitude(apfolder))
      summeandiffamp += meandiffamp
   endfor

   Variable amplitudestd = sqrt((1/(numberDFwaves-1)) * summeandiffamp)
   return amplitudestd
End
```

KINETICS

```
#pragma rtGlobals=3     // Use modern global access method and strict wave access.
#include "Tools window"

Function avrgCurveFit()
   //get wave trace name and display average graph
   String veryFirstWave_name = getVideoName() + "_average"
   Wave averageWave = $veryFirstWave_name
   Display averageWave

   //calculate wavemax
   variable startOffSet = WaveMax(averageWave)

   //find point value corresponding to the max value and find corresponding x value in current scale
   FindLevel /Q/R=[45,170]  averageWave, startOffSet
   variable pointMax = x2pnt(averageWave, V_LevelX)
   variable xMax = pnt2x(averageWave, pointMax)

   //create cursor on designed max point and define last point
   Cursor A $veryFirstWave_name xMax
   variable lastPoint = numpnts(averageWave) - 5

   //calculates curvefit until last point number
   CurveFit/M=2/W=0 exp_XOffset, averageWave[pcsr(A),lastPoint]/D
   PrintKinetics("OffKinetics.txt")
End

Function PrintKinetics(filename)
   string filename
   Close /A
   wave Std_wave = W_sigma
   variable y0Std = Std_wave[0]
   variable AStd = Std_wave[1]
   variable invTauStd = Std_wave[2]

   wave CoefWave = W_coef
   variable y0Coef = CoefWave[0]
   variable ACoef= CoefWave[1]
   variable invTauCoef = CoefWave[2]

   String nameAPs = GetDataFolder(0)
   variable refNum=0
   Open /P=home/A refNum as filename
   fprintf refNum, "%s\t %s\t %f\t %f\t %f\t %f\t %f\t %f\t\r",  getVideoName(), nameAPs, y0Coef, y0Std,
ACoef, AStd, invTauCoef, invTauStd
   Close /A
End
```

Results

SyjRCaMP1b expression in cortical cultures

We did not perform immunocytochemistry to analyse its targeting, however we did a preliminary experiment on its basal fluorescence to study SyjRCaMP1b expression. Taking into consideration not only that no neuronal or synaptic marker was present but also the fact that it was tested in cortical neurons, no conclusions on SyjRCaMP1b expression and targeting in hippocampal cultures can be given. The only parameter of SyjRCaMP1b that can be assayed from figure 7.6 is regarding its basal fluorescence in cortical cultures.

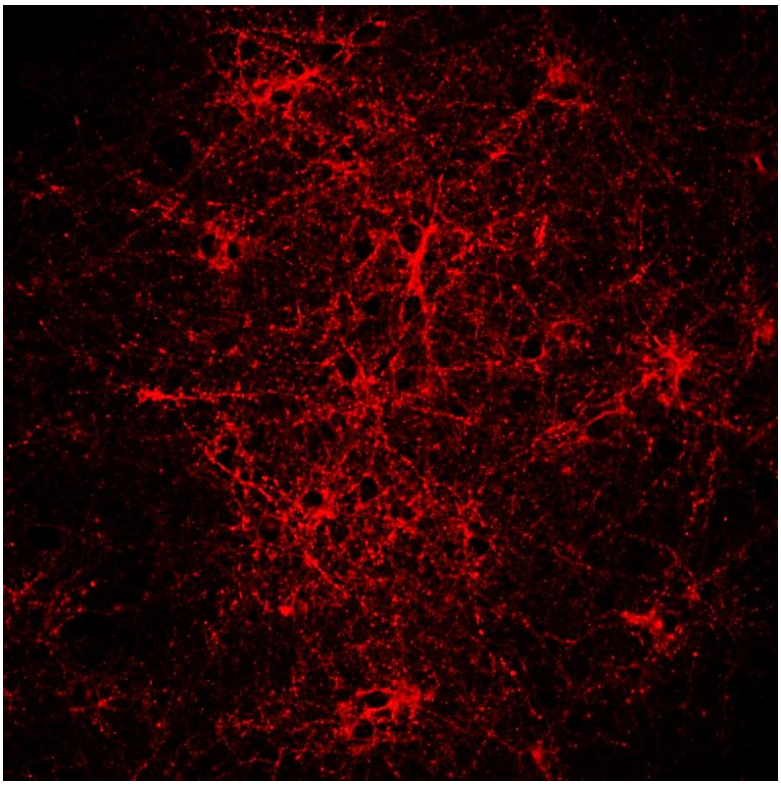

Figure 7.6 – Overview of SyjRCaMP1b basal fluorescence in cortical neurons. Basal fluorescence of SyjRCaMP1b in a well of 96 well plate. Transduction at DIV7 using MOI 100 and cell density of 25000. Pictures were taken using Opera Phenix, with a 40x objective, and processed using Harmony 4.1 software. For RFP, fluorescence was excited using 561 nm laser and emission was collected using 570-630nm filter.

H2B-GCaMP6f characterization

Amplitude

The absolute values of the average peak amplitudes from H2B-GCaMP6f dynamic range graphs were exported to a table (Table 7.1).

Table 7.1 – Absolute values of H2B-GCaMP6f peak amplitudes in the presence and absence of synaptic blockers. Values taken from H2B-GCaMP6f dynamic range graphs in the presence (Blockers) and the absence (No Blockers) of synaptic blockers. In the presence of synaptic blockers, the value amplitude is an average of 68 cell bodies and in the absence of synaptic blockers the value of the amplitude is an average of 13 cell bodies. Amplitude values were calculated using the script.

#APs	$\Delta F/F_0$	
	Blockers	No Blockers
1AP	0.0149	0.1426
2AP	0.0260	0.0412
3AP	0.0523	0.1417
4AP	0.0912	0.2953
5AP	0.1720	0.2958
8AP	0.4976	0.4871
10AP	0.6282	0.3111
20AP	0.8625	0.6861
40AP	1.8148	1.8926

Kinetics

As part of H2B-GCaMP6f characterization, its decay time response was studied in the presence and absence of synaptic blockers for responses from 1 to 40AP (Table 7.2).

Table 7.2- K_{off} constants, in seconds, of H2B-GCaMP6f in the presence and absence of synaptic blockers. Values of K_{off} taken from the H2B-GCaMP6f dynamic range graphs in the presence (Blockers) and absence (No Blockers) of synaptic blockers. In the presence of synaptic blockers, the decay time is an average of 68 cell bodies and in the absence of synaptic blockers the decay time is an average of 13 cell bodies. Values calculated using the script.

#APs	Blockers TauCoef ± SD	No Blockers TauCoef ± SD
1AP	0.94095± 0.016389	1.0335 ± 0.0178
2AP	0.64572±0.049287	0.82591 ± 0.0199
3AP	0.70986±0.023301	0.90775 ± 0.0114
4AP	0.71753±0.021763	2.1072 ± 0.0432
5AP	0.78414±0.0078665	0.90731 ± 0.0069
8AP	0.95849±0.0080641	3.8536 ± 0.0844
10AP	0.799±0.0087312	1.3405 ± 0.055
20AP	0.94134±0.0063159	0.90869 ± 0.0481
40AP	1.1942±0.011033	1.0511 ± 0.0264

"Red Interferences" in H2B-GCaMP6f

Three different regions of one coverslip at DIV16 transduced only with H2B-GCaMP6f was excited at 555nm LED and stimulated with a train of **10AP** at 20Hz, to confirm that there was no photoactivation of H2B-GCaMP6f with the SyjRGECO1a excitation wavelength. H2B-GCaMP6f transduced cultures do not show any significant response in red spectra range (Figure 7.7).

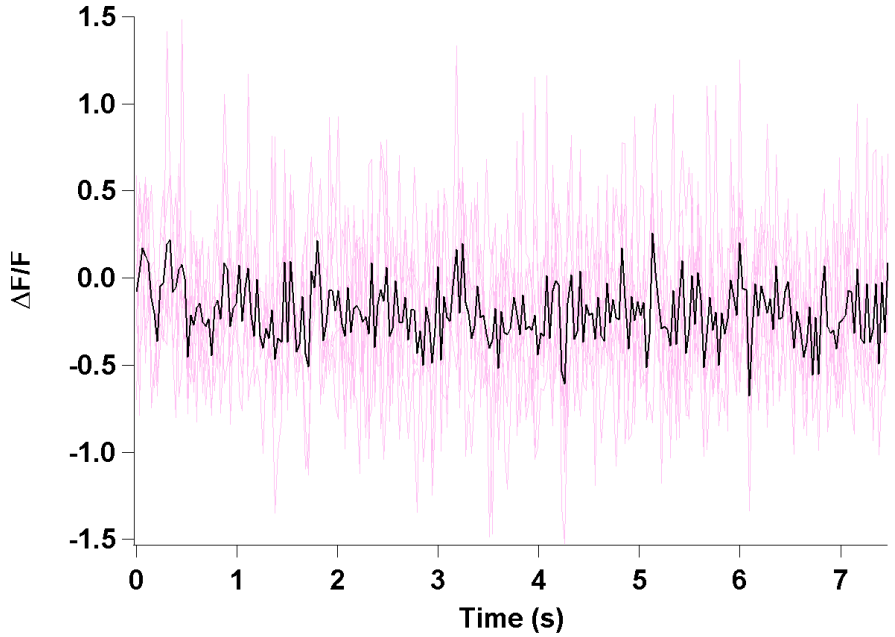

Figure 7.7 – Average response to 10AP at 20Hz, excited with 555nm LED, of neurons transduced only with H2B-GCaMP6f. To test interferences on red spectra in cultures only transduced with H2B-GCaMP6f, 10AP stimulation was delivered. Importantly, each trace does not represent an H2B-GCaMP6f ROI but any response in the neuronal culture

in general. The graph shows an average of 6 waves reactive to the excitation and stimulation.