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To cite this version:
Pauline Campos, Jamie Walker, Patrice Mollard. Diving into the brain: deep-brain imaging techniques in conscious animals. Journal of Endocrinology, 2020, 10.1530/JOE-20-0028. hal-02863790

HAL Id: hal-02863790
https://hal.science/hal-02863790
Submitted on 17 Dec 2020
Diving into the brain: deep-brain imaging techniques in conscious animals

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Running Head: Deep-brain imaging techniques in conscious rodents.

Keywords: Neuroendocrinology, Whole animal physiology, Deep-brain imaging, Neuronal activity, Conscious animals
In most species, survival relies on the hypothalamic control of endocrine axes that regulate critical functions such as reproduction, growth, and metabolism. For decades, the complexity and inaccessibility of the hypothalamic-pituitary axis has prevented researchers from elucidating the relationship between the activity of endocrine hypothalamic neurons and pituitary hormone secretion. Indeed, the study of central control of endocrine function has been largely dominated by ‘traditional’ techniques that consist of studying in vitro or ex vivo isolated cell types without taking into account the complexity of regulatory mechanisms at the level of the brain, pituitary and periphery. Nowadays, by exploiting modern neuronal transfection and imaging techniques, it is possible to study hypothalamic neuron activity in situ, in real time, and in conscious animals. Deep-brain imaging of calcium activity can be performed through gradient-index lenses that are chronically implanted and offer a ‘window into the brain’ to image multiple neurons at single-cell resolution. With this review, we aim to highlight deep-brain imaging techniques that enable the study of neuroendocrine neurons in awake animals whilst maintaining the integrity of regulatory loops between the brain, pituitary and peripheral glands. Furthermore, to assist researchers in setting up these techniques, we discuss the equipment required, and include a practical step-by-step guide to performing these deep-brain imaging studies.
For almost a century, neuroendocrinologists have worked towards characterising the central regulation of endocrine axes that ensure critical functions such as reproduction, growth, and metabolism. While research questions have for many years focused firmly on the neural control of the pituitary gland, the neuroendocrinology field has grown wider and now includes studying the effect of centrally-produced hormones on various brain areas, as well as the role of several peripherally-born peptides that affect neuroendocrine systems controlling metabolism. An unavoidable feature of all neuroendocrine systems is that they generate rhythms and rely on these rhythms to function optimally. These hormone oscillations dynamically regulate gene transcription and synaptic transmission, and changes in these oscillations are observed in a variety of (patho)physiological states (Le Tissier et al., 2017).

Although it has long been postulated that neuroendocrine neurons have the ability to generate hormone rhythms via the patterning of their electrophysiological activity, the regulatory mechanisms underlying this rhythmic mode of hormone secretion have remained hazy. One reason for this is that research efforts in this area have simply been hindered by the complexity and inaccessibility of the hypothalamic-pituitary system. Another reason is that the study of neuroendocrine function has been largely dominated by techniques that consist of studying isolated cell types in in vitro or ex vivo preparations. This insular approach has led to an inevitable bottleneck where data on cellular and biochemical processes within specific cell types or nuclei have multiplied without a clear/tangible link to physiological function. Similarly, recent efforts in single-cell transcriptomics of the hypothalamus (Romanov et al., 2017; Wang and Ma, 2019) have documented a highly complex heterogenous hypothalamus, but the implication of neuropeptide expression for physiological function remains difficult to interpret without appropriate tools. Nevertheless, technological developments in genetics and systems neuroscience have enabled specific neurons to be manipulated in vivo. Chemogenetics and optogenetics (Fenno et al., 2011) tools have made it possible to study the hypothalamus in ways that were previously unimaginable, shedding light on gonadotropin pulse generation (Campos and Herbison, 2014; Han et al., 2015; Voliotis et al., 2019) and brain control of appetite for example (Atasoy et al., 2014).
2012; Betley et al., 2015). Likewise, genetically-encoded calcium indicators (GECIs) (Pérez Koldenkova and Nagai, 2013) are now frequently used to monitor neuronal activity in living animals. Their characteristics making them an excellent proxy for electrical activity and a versatile tool that facilitates diverse imaging techniques. For example, fibre photometry experiments that consist of monitoring the average calcium activity of a neuronal population in vivo have shown that arcuate kisspeptin neurons are responsible for the generation of pulses of LH (Clarkson et al., 2017).

Despite the valuable insights these techniques have provided, none of them have allowed researchers to study neuronal activity across a population at the single-cell level—characterising for example cell-to-cell heterogeneity or synchronicity—and in turn relate this activity to specific functions. Fortunately, deep-brain single-cell imaging can now be achieved using gradient-index (GRIN) lenses that are chronically implanted and permit imaging of multiple (10-100s) neurons within the population (Barretto et al., 2009). Depending on the research question and image quality required, visualisation of neuronal activity can be carried out in head-fixed configuration using a bench-top microscope (Bocarsly et al., 2015; Kim et al., 2010, 2009), or in freely-moving configuration using a miniature head-mounted microscope (Ghosh et al., 2011). Importantly, using these techniques, it becomes possible to correlate the impact of neuronal activity within a network on other functions. This methodology has been successfully used to study arcuate nucleus and amygdala control of feeding behaviour in freely-moving mice (Betley et al., 2015; Jennings et al., 2015). Similarly, head-fixed microscopy through GRIN lenses has made possible to image and manipulate pituitary cells over a period of days to weeks in awake mice (Hoa et al., 2019) and while it has yet to be published, one can easily imagine combining this technique with serial blood sampling to understand the link between the activity of specific hypothalamic neurons and the resulting peripheral hormonal release (Fig1). In the near future, in vivo deep-brain imaging could become critical when combined with other emerging strategies. For example, researchers now have the ability to visualise in vivo the effect on neuronal activity of individual genomic variants identified from human genomes, thus filling a gap in our general view of neuroendocrine systems (Fig1).

In this review, we will discuss deep-brain imaging techniques that enable the study of endogenous brain activity from neuroendocrine populations in awake animals whilst maintaining the integrity of regulatory loops between the brain, pituitary and peripheral glands. Rather than cataloguing all available methodologies, we will focus on the most widely used and accessible systems, and provide advice on neuronal activity indicators, neuronal targeting, microscopy techniques, analysis, animal handling and physiological/behavioural assessments so that researchers will be able to set up these techniques to address their own research questions.
1. Monitoring neuronal activity with genetically-encoded indicators

In order to understand brain processing, scientists have tried to decrypt the action potential (AP) firing of groups of neurons and relate this to specific physiological functions. Traditional methods such as intracellular patch-clamp limit the number of neurons that can be studied at the same time, are invasive and difficult to perform \textit{in vivo}. Extracellular recordings are easier to make, but do not give precise information about the electrical activity of individual neurons, and do not enable the study of specific neuronal firing within a population. Looking to overcome these issues, Francis Crick proposed in 1999 that “One way-out suggestion is to engineer these neurons so that when one of them fires it would emit a flash of light of a particular wave length” (Crick, 1999); and indeed, the development of genetically-encoded optical indicators of neuronal activity has enabled progress toward this goal to an extent that was unimaginable two decades ago. Nowadays, optical imaging enables thousands of neurons to be simultaneously observed \textit{in vitro} or \textit{in vivo}, whilst voltage and calcium indicators reveal spatiotemporal activity patterns within neurons such as dendritic integration, voltage propagation, or dendritic spiking (Lin and Schnitzer, 2016). Although microscopy requires optical access to the tissue of interest, many techniques allow minimally-invasive optical access or placement of optical probes up to millimetres away from the imaged cells, thus allowing the immediate vicinity of cells under study to be left unperturbed (Betley et al., 2015; Ghosh et al., 2011).

a. Calcium indicators

Calcium is a universal second messenger, playing an essential role in excitable cells and signal transduction. In neurons, APs trigger large and rapid calcium (Ca$^{2+}$) influx through voltage-gated channels. AP firing can therefore be assessed by measuring changes in concentrations of intracellular Ca$^{2+}$ (Yasuda et al., 2004). Similarly, activation of neurotransmitter receptors during synaptic transmission causes Ca$^{2+}$ transients in dendritic spines, and with appropriate indicators, imaging intracellular Ca$^{2+}$ dynamics can be used to measure neuronal spiking and synaptic activity across populations of neurons \textit{in vitro} and \textit{in vivo}. GECIs and small-molecule calcium-sensitive dyes (Cobbold and Rink, 1987) such as fura-2 (Gryniewicz et al., 1985) are both used to report changes in intracellular Ca$^{2+}$ concentration; but GECIs have the advantage that they enable long term, repeated non-invasive imaging of specific cells and compartments (Mao et al., 2008). State-of-the-art GECIs include Förster Resonance Energy Transfer (FRET)-based sensors (Fiala et al., 2002; Mank et al., 2008; Palmer and Tsien, 2006), but for \textit{in vivo} experiments particularly, the single-wavelength sensor GCaMP family has become the default tool (Tian et al., 2009). GCaMPs are based on circularly permuted green fluorescent protein (cpGFP), calmodulin (CaM), and the Ca$^{2+}$/CaM-binding “M13” peptide. Ca$^{2+}$ binding to the calmodulin domain results in a structural shift that enhance the fluorescence by deprotonation of the fluorophore. Since the original GCaMP sensor was published (Nakai et al., 2001), many variants have
been developed with the purpose of performing calcium imaging in vivo (Ohkura et al., 2005; Tallini et al., 2006; Tian et al., 2009). The experimenter will be burdened with picking the appropriate GECI from a broad assortment, but generally-speaking, imaging from large populations of neurons in densely labelled samples is helped by lower baseline fluorescence indicators, which reduces the background signal from neuropil and inactive neurons (Dana et al., 2019). Moreover, low baseline fluorescence becomes especially important when working in vivo where large tissue volumes contribute to the background signal. For the reasons mentioned above, we and others have worked with GCaMP6s,m,f and are now experimenting with JGCaMP7f (Chen et al., 2013); all offer high signal-to-noise ratio (SNR) and GCaMP6m and JGCaMP7 show improved detection of individual spikes (Chen et al., 2013; Dana et al., 2019). It is also worth noting the development of red-shifted GECIs (e.g. jRCaMP1a, jRCaMP1b, jRGECO1a, jRGECO1b) (Akerboom et al., 2013; Dana et al., 2016; Montagni et al., 2019); these allow the simultaneous use of optogenetic activation of Channelrhodopsins using blue light while recording calcium activity (Akerboom et al., 2013).

b. Voltage indicators

Voltage imaging can directly display membrane potential dynamics and has been used to measure neural dynamics for decades (Bando et al., 2019). Genetically-encoded voltage indicators (GEVIs) can be categorised into 3 classes based on their molecular structure and voltage-sensing mechanism: voltage-sensitive domain-based sensors, rhodopsin-based sensors, and rhodopsin-FRET sensors (see (Bando et al., 2019) for a comparative review on GEVIs). While imaging voltage using fluorescent-based sensors appears to be an ideal technique to probe neural circuits with high spatiotemporal resolution, due to insufficient SNR, imaging membrane potential is still challenging, and in order to achieve single action potential resolution in vivo, brighter voltage indicators will need to be developed (Aharoni and Hoogland, 2019).

2. Directing GECIs to neurons of interest

a. Viral Vectors

Viruses can be used to target Ca^{2+} indicators to specific cells in vivo. Adeno-associated virus (AAV) and lentivirus (LV) are the most common viral vectors used today for neuroscience applications and result in robust expression of the desired gene throughout the cell. Due to their low immunogenicity, AAVs and LVs are well tolerated and highly expressed over long periods of time without any reported adverse effects (Kaplitt et al., 1994; Sakuma et al., 2012; Samulski and Muzyczka, 2014) in various
species (Vasileva and Jessberger, 2005). AAVs are the most widely used by the scientific community; their efficiency has been evaluated for many brain regions and cell types, and most of them are commercially available from facilities such as Addgene, Penn Vector Core, or ETH Zurich. It is very common to deliver a Cre-dependant viral vector in a Cre-line mutant mouse. With this approach, Ca$^{2+}$ indicator cell-type specificity is typically determined by the genetically-controlled presence of a recombinase (Cre) in the specific cells of interest. The viral approach has several advantages, including topographic specificity and the ability to control the amount of protein transduced (adjustment of volume and concentration of viral vectors). However, viral neuronal transfection relies on stereotaxic injection and therefore every animal is unique. Viral vectors also have to be carefully selected and require rigorous post-hoc histological validation.

**AAVs**

AAVs are small particles (20nm) allowing them to spread very efficiently within tissues and are particularly well-adapted to brain cell transfection. The choice of the appropriate AAV needs be based on multiple factors, from the physical location of the viral injection in the brain, to the serotype/cell-type affinity of the chosen viral vector, as well as the molecular and genetic machinery driving its replication/integration. AAVs present different serotypes meaning that their capsid proteins differ as well as DNA sequences (inverted terminal repeats, ITRs) that result in variations in speed of viral gene expression onset and target cell specificity. Overall, AAV1, 2, 5, 6, 8, and 9 have different affinities for different cells but should all be able to transfect neurons (Aschauer et al., 2013; Hammond et al., 2017). When in doubt, AAV-DJ (Grimm et al., 2008) or AAV DJ8 (Hashimoto et al., 2017) may be good options to test out transfection of neurons that have not been studied before due to their alleged increased tissue tropism. Several ubiquitous promoters are available to drive transgene expressions with AAVs; although not exhaustive, Table 1 summarizes serotypes and promoters used to successfully transfect various hypothalamic neuronal populations.

**Lentiviruses**

LVs are big particles (80-120nm) and can carry inserts as large as 10kb (as opposed to the limited 4.5 kb capacity of AAVs), which is particularly pertinent when working with a promoter-specific virus. Although they are less commonly used than AAVs, LVs are more efficient at transfecting cells in vitro, have similar capabilities in vivo (but do not spread as widely as AAVs) and do not require making a choice of serotype (Cannon et al., 2011).
b. Transgenics

In order to work with animals with similar levels of transgene expression within the targeted neuronal phenotype, transgenic lines have been generated. Essentially, two approaches exist.

In the first type of transgenic line, GCaMP is expressed under the control of specific promoters. For example, the Thy1-GCaMP3 (Chen et al., 2012), 6s and 6f (Dana et al., 2014) transgenic mouse lines express GCaMP in Thy1-expressing neurons across the entire brain and have been used for in vitro and in vivo Ca\(^{2+}\) imaging. Similarly, GCaMP2 expression under the Kv3.1 potassium-channel promoter has been used to perform Ca\(^{2+}\) imaging of the glomerular layer of the mouse olfactory bulb (Fletcher et al., 2009). Finally, transgenic rat lines have been developed and Thy1-GCaMP6f rats have been used to record cortical activity with a head-mounted microscope (Scott et al., 2018).

The second and more common approach is to generate transgenic animals bearing GCaMP expression in specific cell types, by crossing a Cre-driver with a floxed-GCaMP mouse (Hsiang et al., 2017; Zimmerman et al., 2019). Several floxed-GCaMP mice lines are now commercially available from Jackson laboratories and have been characterised by users. Nevertheless, potential concerns have to be kept in mind if working with transgenics and attention will need to be given to potential ectopic expression, drift and loss of specificity of the transgene, or even disrupted Ca\(^{2+}\) dynamics within GCaMP-expressing neurons (Steinmetz et al., 2017). After years of working with GECIs or genetically-addressable opsins, many labs including our own have favoured the use of viruses. Overall, viral vectors lead to a greater level of expression of the desire transgene, which is essential in vivo, and generating transgenic lines has been an expensive, time-consuming process that has not always resulted in success.

c. Histological validation of GCaMP expression

Regardless of the technique used to target GCaMP to specific cells, it is essential to confirm the extent of transgene expression, spread, and location over the experimental timeline using histology methodologies. Typically, animals will be terminally anesthetized and transcardially perfused with a 4% paraformaldehyde solution, and brains will be removed, sectioned and processed for histological analysis. Native GCaMP expression should be bright enough to visualize in fixed sections with epifluorescence or confocal microscopy, with fluorescence signal limited to the cell membrane. Filled nuclei and/or misshapen neurons can be a sign of overexpression, which can be overcome by reducing viral vector concentration.

In order to identify transfected neurons with immunohistochemistry techniques, GCaMP labelling can be achieved using a GFP antibody; this is especially useful in situations where GCaMP cannot be clearly detected without enhancement. Alternatively, it is possible to perfuse the animal with calcium-containing DPBS (Dulbecco’s Phosphate Buffer Saline). Since DPBS contains a saturating
concentration of Ca\(^{2+}\) (0.9 mM), GCaMP brightness will be maximal, and the experimenter may therefore be able to visualize GCaMP fluorescence without antibodies (Dana et al., 2014).

3. Imaging deep brain areas

While cortical activity can be recorded through cranial window, hypothalamic neuroendocrine neurons are located deep in the brain (from half a centimetre deep for mice to up to a centimetre deep for rats) and it is therefore necessary to find tools that allow the experimenter to see ‘deep inside’ the brain. Fortunately, in parallel to the development of Ca\(^{2+}\) indicators, instrumentation was optimized to render Ca\(^{2+}\) imaging possible in subcortical brain areas.

a. GRIN lenses

GRIN lenses resemble simple glass needles, but they are in fact complex lenses with a radially-varying index of refraction; this causes an optical ray to follow a sinusoidal propagation path through the lens. GRIN lenses combine refraction at the end surfaces along with continuous refraction within the lens. Their characteristics allow them to relay optical images from one end to the other, with very low aberrations for objects near the optical axis. For these reasons, GRIN lenses are the perfect tool to perform in vivo imaging in deep-brain regions, relaying signals a point above the skull surface where image acquisition can take place (Barretto et al., 2009). GRIN lenses exist in different lengths and diameters. For brain regions such as the dorsal striatum (Zhang et al., 2019), prelimbic cortex (Pinto and Dan, 2015) or hippocampus (Ziv et al., 2013), a 1 mm diameter GRIN lens can be used to increase the field of view. For deep-brain areas such as the amygdala (Li et al., 2017) or hypothalamus (Jennings et al., 2015), a lens of approximately 0.5mm diameter can be used in order to minimize tissue damage during lens implantation. For neuroendocrine hypothalamic areas (e.g. arcuate nucleus, paraventricular nucleus, rostral preoptic area), we have successfully used 0.6mm diameter 7.4mm long lenses for mice, and 0.8mm diameter 10.8mm long lenses for rats.

b. Prism probes

Side-view GRIN lenses or ‘prism-probes’ exist. These are composed of a prism fixed to the bottom of a cylindrical GRIN relay lens. The slanted surface of the prism is coated with metal (aluminium or silver for high reflectivity) to reflect by 90° the light from the microscope to excite GCaMP cells located along the imaging face of the prism probe. The emitted light from the cells also reflects off the hypotenuse of the prism and can be collected through the objective of the microscope. These probes
have been used for multi-layer cortical imaging (Gulati et al., 2017) and could be suitable for populations of hypothalamic neurons that are spatially distributed along the z-axis.

c. GRIN lens implantation

Unravelling the intricacies of neuroendocrine function with in vivo imaging studies lasting days to weeks requires optical access to the structure of interest while maintaining both its integrity and that of the surrounding tissue. First of all, before performing GRIN lens implantation in animals, it is sensible to use a ‘phantom brain’ preparation where the GRIN lens is implanted into an agar/fluorescent microbead preparation (Kim et al., 2017). This preparation can be looked at under any epifluorescence microscope in order to verify the working distance of the GRIN lens. GRIN lenses have to be placed above the targeted structure and the space needed between the lens and the neurons is determined by the working distance (50-350µm) of the imaging side of the GRIN.

In order to gain optical access to the hypothalamus, a GRIN lens has to be guided through the cortex towards the ventral side of the brain. To overcome the challenge of crossing the meninges and going through the brain tissue without increasing pressure and causing excessive damage, the GRIN lens can be inserted into the lumen of a needle, which can then be retracted once the GRIN lens is located correctly. Practically, the GRIN lens is placed inside a small gauge needle, with movement along the needle restricted by placing a metal rod above it. The needle is then lowered to a point just above the target, and then removed with the metal rod kept in place, ensuring the GRIN lens stays in place. Finally, the metal rod is removed and the GRIN lens fixed in position with dental cement (Hoa et al., 2019). An alternative is to first create a path to the target structure with a needle or a dissection knife (Gulati et al., 2017), and to then lower the naked GRIN lens along this path; in this method, the lens can be carefully held using a bulldog clamp or a pipette tip trimmed to match the diameter of the lens. Another interesting method consists of placing the top part of the GRIN lens inside a glass tube and fixing it using Super Glue. The glass tube can then be held with an electrode holder on the stereotaxic arm. Once the GRIN lens has been positioned in the brain and cemented in place, acetone can be carefully introduced into the glass tube in order to dissolve the Super Glue, thus freeing the GRIN lens (Zhang et al., 2019).

GRIN lens implantations are invasive procedures that cause inflammatory responses in the brain, during which the neurons do not behave normally. It is recommended to wait at least 3 weeks after implantation before starting imaging sessions; overall, we have had the most success imaging Ca^{2+} activity from 5 weeks to several months after GRIN lens implantation. It is also necessary to ensure that GRIN lens implantations do not result in long-term inflammatory responses that could alter physiological function.
Attention therefore needs to be given to possible behavioural/hormonal changes, and post hoc histological validation is necessary.

d. **Sourcing GRIN lens**

Currently, to our knowledge, there are two manufacturers of GRIN lenses suited to brain imaging applications. The first is GRINTECH, who hold a strategic partnership with the neurotechnology company Inscopix. All GRINTECH lenses used in the field of non-confocal, single photon epi-fluorescence imaging for non-human neuroscience application must be purchased through Inscopix. The second manufacturer is GoFoton, who are the largest manufacturer of GRIN lenses in the world. Although their GRIN lens production has been focused on telecommunication applications and fibre coupling, they have been working over the past year at developing GRIN lenses specifically for brain imaging applications. Hopefully, GoFoton will be able to provide high quality GRIN lenses for microscopic applications in the near future.

4. **Imaging in conscious animals**

Deep-brain imaging of Ca$^{2+}$ activity can be performed through GRIN lenses that are chronically implanted; these relay fluorescence captured inside the brain to the surface of the skull, which can be observed with conventional microscopy techniques. Depending on the research question and the image quality needed, deep-brain imaging can be performed with a bench top microscope on head-restrained animals or with the help of a head-mounted miniature microscope for freely-moving animals.

a. **Head-fixed configuration**

In order to perform deep-brain imaging in conscious animals, rodents can be temporarily restrained under a microscope with the help of a metal piece chronically fixed to their skull. Animals retain the ability to move on a wheel or a cylinder in order to give an impression of movement and reduce stress during the experiments. This approach has been used to perform calcium imaging in deep-brain areas (Bocarsly et al., 2015), and even in two areas at the same time (Lecoq et al., 2014). While most of these studies have been performed using two-photon microscopes, we have successfully performed 1-photon calcium imaging in the hypothalamus of head-fixed mice with a stereomicroscope fitted with a 20x objective (for detailed methodology see (Hoa et al., 2019)). Using this set-up, we investigated the endogenous activity of hypophysiotropic TRH neurons across several days. To do so, we worked with TRH-Cre mice (Krashes et al., 2014) (kindly gifted by Pr. B. Lowell, Beth Israel Deaconess Medical Center & Harvard Medical School) that had been injected with a Cre-dependant AAV driving GCamp6s expression. We recorded the activity of several TRH neurons at single-cell resolution level in head-
fixed mice (Fig2). This experimental design enabled us to study how hypophysiotropic TRH neurons interact with each other within local networks and modulate their activity throughout the day (Campos and Mollard, unpublished observations). A great benefit of deep-brain imaging in head-fixed configuration is that bench top microscopes offer excellent imaging quality and it is therefore possible to record the activity of sub-cellular structures (Meng et al., 2019). Indeed, we have successfully imaged the calcium activity of assemblies of TRH neuron terminals in the median eminence, although 1-photon microscopy could not spatially resolve individual boutons (Campos and Mollard, unpublished observations).

b. Miniscopes

In 2011, Mark Schnitzer’s group first reported the miniature microscope (miniscope) with epifluorescent light source (Ghosh et al., 2011), which was small, light, and easy to mount on the head of a mouse for studying complex behaviours in freely-moving animals. Combined with a GRIN lens, the miniscope imaging system enables recording of calcium activity from hundreds of neurons in deep-brain regions for months, with imaging quality comparable to benchtop microscopes. Over the last 5-10 years, several miniscope designs have emerged, but currently, two miniscopes in particular appear to be widely used: the Inscopix nVista miniscope and the UCLA miniscope. These two miniature microscopes share the same core features. The optical design includes an achromatic lens which focuses near-collimated light from an objective GRIN lens onto a CMOS sensor and a set of optical filters. The plastic housing can be machined or 3D printed, and custom electronics are used to control and read-out the excitation LED light source and CMOS imaging sensor. Miniscopes are connected to a data acquisition (DAQ) box that relays data to a computer. A base plate is attached to the skull of the animal with dental cement and allows magnetic latching of the miniscope to the baseplate.

nVista Inscopix Miniscope.

The Inscopix company was founded in 2011 by Kunal Ghosh, Mark Schnitzer and Abbas El Gamal in order to commercialise their miniature microscope. Some years later, their technology has evolved and the Inscopix miniature microscope, called nVista, is now widely used. The characteristics of the current version of the nVista microscope are presented in Table 2. It possesses a software-controlled electronic focusing mechanism (300 μm) that does not require any manual intervention. The miniscope is connected to a DAQ box which has the capacity to store data on an SSD and/or an SD card. The DAQ box is also equipped with ethernet and WIFI connectivity, which allows remote control of the miniscope and data streaming through web-based acquisition software.

On purchasing the nVista system, researchers will receive a full support package, which makes it probably the easiest way to start working with deep-brain imaging with a miniscope. Researchers will
benefit from field scientific consultants that will, through site visits and online communication, help them set up the technology and troubleshoot experimental and conceptual challenges (from Ca²⁺ indicator selection, to data analysis) in order to ensure the success of research project. It is worth highlighting that the nVista miniscope comes with a 1-year manufacturer’s warranty that can be prolonged to 5 years. For damaged scopes sent back to the company, turn-around time is typically around 1 week. In our own experience, this warranty has been valuable; we have had to return damaged nVista minisscopes to Inscopix 3-4 times per year for repair. This support package comes at a price of course; currently, the nVista system (consisting of one nVista 3.0 scope + accessories + 1-year warranty + help + software) costs in the region of 70-80000 USD.

UCLA Miniscope

The UCLA miniscope is also based on the design pioneered by Mark Schnitzer's Lab, but this miniscope is open-source. The characteristics of the UCLA miniscope version 3 (V3) are presented in Table 2. It also uses a single, flexible coaxial cable to carry power, communication, and data transmission to a custom open source DAQ box and software. The default version of the UCLA miniscope only allows cortical imaging. For imaging deeper brain areas, an objective GRIN lens can be mounted inside the miniscope and positioned above a GRIN relay lens chronically implanted above the target cells. Focusing adjustment (300μm) occurs through a linear slider that can be manually adjusted in height; although it preserves the orientation of neurons between imaging sessions, it can be tricky to use, especially when the miniscope is head-mounted.

The UCLA Miniscope has had a significant impact on the neuroscience community, with at least 400 labs around the world building and using these imaging devices for their research studies over the past 3 years. This success is likely due to a broad dissemination policy. The ‘miniscope project’ began as a collaboration between the Golshani Lab, Silva Lab, and Khakh Lab at the University of California, Los Angeles, and provides documentation, tutorials on part procurement, assembly and experimental application guides, both online and through workshops. The http://miniscope.org website provides a centralized hub for users to share design files, source code, and other relevant information related to this imaging technique.

Building a UCLA miniscope is cost-effective (DAQ + 1 scope < $1,000 USD). It is not complicated but does require soldering and hands-on assembly. A main advantage is that it can be tuned to fit the research project requirements, and additionally by building it, researchers understand how to fix it quickly. For those that do not feel confident building the miniscope, LabMaker (www.labmaker.org), based in Berlin, Germany, sell the UCLA miniscope as a kit that does not require any soldering for approximately $2,000 USD (includes DAQ + 1 scope).
Improvements of the UCLA miniscope

It is worth noting that this is a rapidly-evolving field, with technological improvements in miniature microscopes emerging all the time. For example, at the time of writing, a new version (V4) of the UCLA miniscope has just been released (http://miniscope.org). Compared to the UCLA V3 miniscope, the V4 miniscope is lighter (2.6g), utilises a smaller baseplate (25 μm footprint), has a field of view that can be set based on the combination of lenses used when assembling the miniscope (1000 μm-diameter or more), has an improved CMOS that requires just a fifth of the excitation power of previous systems, and is expected to have improved resolution (0.5MP) and faster imaging speed (120FPS) (Daniel Aharoni, 2019 Neuro Open-source Workshop, January 2019). In addition, the manual focusing mechanism of V3 has been replaced by an electronic system. Indeed, similar to the nVista system, the V4 miniscope benefits from liquid lenses that consist of an interface of two immiscible liquids which, under voltage, deform and act as a lens allowing for electronic focusing without the need for manual adjustment. This should make focusing much easier, and because liquid lenses can be adjusted rapidly, it may become possible to perform interleaved recording from different focal planes (Aharoni and Hoogland, 2019). Finally, in order to allow for simultaneous two-colour fluorescence imaging, the objective GRIN lenses present in the previous version of the UCLA miniscopes that created chromatic aberrations, have been replaced in V4 miniscopes by achromatic lenses (Aharoni and Hoogland, 2019). However, since deep-brain imaging necessitates the implantation of GRIN lenses in the brain, this new feature does not yet represent an advantage for the study of deeply-located neuroendocrine cells. It is important to note that although the UCLA V4 miniscope possesses promising improvements and can be fitted with relay GRIN lenses for deep brain imaging, to our knowledge, they have not yet been tested for structures deeper than the cortex. The V4 is expected to be released as a kit in 2020.

c. Alternatives and next generation miniscopes

Wire-free scopes

Whilst tethered systems are currently the most effective method for in vivo recording/control of freely-moving animals, wireless (Liberti et al., 2017) and wire-free (Shuman et al., 2018) open-source miniscopes have been developed with the latest being used to record CA1 place cells during maze navigation in epileptic mice. These recent studies have demonstrated the feasibility of using a lightweight wireless miniature microscope system to transmit Ca2+ imaging data to a receiver (Liberti et al., 2017) or save data directly to a microSD card positioned on the miniscope (Shuman et al., 2018). Both designs are powered by onboard lithium polymer batteries that can be attached to the microscope body or mounted on the back of the animal, consequently adding weight to the microscope design (~2...
g for a 50 mAh battery). Alternatively, wireless powering of a miniaturized design with low power consumption is thought to be feasible with near-field wireless power transfer similar to those that have already been implemented for wireless optogenetic stimulation (Aharoni and Hoogland, 2019). While power consumption (which scales with data bandwidth) is one of the limiting factors in designing a miniaturized wireless microscope due to battery size and weight requirements, power-efficient CMOS imaging sensors can be used along with pixel binning or pixel subsampling to minimize power consumption while maintaining a comparable field of view, albeit at a lower resolution than is possible using tethered systems (Aharoni and Hoogland, 2019). The open-source nature of ongoing miniscope projects allows for the rapid sharing of designs, modifications and ideas across laboratories, and it is expected that wireless systems will rapidly improve and that their use will soon escalate within the neuroscience community. Of particular interest here is Open-ePhys, a non-profit initiative encompassing a large team of scientists and developers, which has released a kit to convert the wired UCLA miniscope (version 3) into a wire-free system.

Two-Photon imaging

Two-photon (2P) excitation improves optical sectioning of samples and thereby ensures that the light collected is only from the cellular and subcellular structures that lie along the focal plane of 2P excitation. While 2P brain imaging in animal models usually requires that the animal is head-fixed, there have been several successful attempts at building a 2P miniscope (see (Helmchen et al., 2013) for review) with some designs used to image in freely-moving rodents (Ozbay et al., 2018; Zong et al., 2017). A major disadvantage to this technique is that it requires expensive table-top lasers coupled to optical fibres to achieve 2P excitation (Aharoni and Hoogland, 2019). Furthermore, a recent study compared data acquired with a 2P benchtop microscope with one-photon images acquired with a miniscope, and found that orientation tuning of the same identified neurons was comparable irrespective of the type of fluorescence excitation used (Glas et al., 2019).

5. Animal handling

Regardless of the imaging technique, it is important to take the time to handle animals to ensure they become familiar with the experimenter and being moved in and out of their cage. It is possible to quickly and briefly anaesthetise the animal when attaching the miniscope or placing the animal in the head-attached set-up, but it is not recommended. Indeed, as discussed later in this review, anaesthetics, even when used for a very short period of time, may affect neuronal dynamics, lead to changes in hormone secretion, or alter animal behaviour.
a. Head-fixed habituation

For head-fixed experiments, it is useful to place the animal under the microscope for a few minutes every day leading up to the experiment. Attention should be paid to details such as lighting, electrical equipment, and ventilation in order for the animal to acclimatise to the exact conditions that will be used for the experiment. It is also important to protect the animal’s eyes from the microscope’s excitation light by covering the front part of the head with suitable opaque material. We have found that animals never get completely used to being head restrained for long periods of time, but studies have reported self-initiated head-fixation system for functional imaging in mice. For example, mice have been shown to self-initiate head-fixation in order to get water, enabling imaging to be performed through cranial windows (Aoki et al., 2017; Murphy et al., 2016). Although it has never been used for deep-brain imaging, this habituation technique seems promising and shows that with patience and good experimental design it is indeed feasible to perform imaging in ‘low stress’ head-fixed animals.

b. Miniscope habituation

For miniscope experiments, the acclimatisation process is more straightforward. Animals should first habituate to their imaging arena for a few minutes each day leading up to the experiment. Then, it is important for the animals to get used to carrying the miniscope, which can weigh about 10% of the weight of an adult mouse. We use ‘dummy’ minisscopes to train our animals; they are of the exact same weight and size as the real miniscope and are connected using the same type of cable. We have found that animals carrying dummies for a few days soon behave in a completely normal way. It is also useful to use a dummy miniscope so the animals get used to the microscope cable. Indeed, mice will try to chew on the cable, and we have found that coating the dummy cable with bitter nail polish usually prevents mice from chewing the real cable during experiments. We have yet to try this methodology with rats.

c. Evaluating stress

Together with handling and habituation, it is recommended to evaluate the level of stress of the animals throughout the experiments by measuring corticosterone concentration. Commercially-available enzyme-linked immunosorbent assay (ELISA) kits are widely used to quantify corticosterone levels for the assessment of stress in laboratory animals. Some of them, such as the AssayMax corticosterone ELISA kit (AssayPro), only require small volumes of whole blood (3-6 µL per sample) that can be collected using a minimally-invasive technique such as tail-tip blood sampling (Guillou et al., 2015). Although the precision in determining true values of corticosterone is low for commercial ELISA kits,
they have proven to be useful to determine relative differences within studies (e.g. head-fixed animals vs. freely-moving animals vs. sham animals).

6. Analysis

In vivo Ca\textsuperscript{2+} imaging using GRIN lenses and 1-photon-based microscopes enables the study of neural activities in behaving animals which would be impossible using other techniques. However, the high and fluctuating background fluorescence, inevitable movement and distortion of the imaging field, and extensive spatial overlaps of fluorescent signals emitted from imaged neurons, present major challenges for extracting neuronal signals from the raw imaging data (Lu et al., 2018). Overall, the experimenter will need to identify the spatial outline of each neuron in the field of view, untwine spatially-overlapping neurons, and finally deconvolve the spiking activity of each neuron from the dynamics of the Ca\textsuperscript{2+} indicator.

a. Enhancement and Motion correction

Overall, several steps are necessary to obtain valuable signals. First, the data needs to be ‘enhanced’ by applying a spatial filter that enhances the signal intensity and removes as much background noise as possible. It is then necessary to perform movement correction in order to align (or register) images. This step not only helps correct for movement that can occur when animals are grooming, chewing etc., but also helps with brain tissue movement in areas close to ventricles. Indeed, we have observed that the brain shows movement even when animals are static, which we hypothesise is due, at least in part, to cerebral spinal fluid flowing through the ventricles. Non-rigid motion-correction algorithms that run on Fiji (such as Moco (Dubbs et al., 2016)) or Matlab (such as NoRMCorre (Pnevmatikakis and Giovannucci, 2017)) have proven useful in solving large-scale image registration problems.

b. Extraction of calcium signals in regions of interest

When images have been treated and motion corrected, they are ready to be analysed. Fluorescence in regions of interest (ROI) can be measured over time. The selection of ROI can easily be done by hand (ROI Manager in Fiji) in very scattered populations, but for large data sets that contain a dense populations of neurons, automated cell-sorting algorithms may be better suited to extract spatial and temporal properties of individual cellular Ca\textsuperscript{2+} signals (Mukamel et al., 2009; Pnevmatikakis et al., 2016). In brief, most cell identification algorithms run principal component analysis (PCA) to
remove signals that mostly encode noise. Dimension reduction is then followed by independent
component analysis (ICA) to identify the spatial and temporal properties of visualized cells by
segmenting the data into statistically-independent spatial and temporal signals. PCA/ICA relies on
statistical independence of signals and fluorescent signals will be identified as cells only if their
activities are not synchronised and if they do not have the same pixel coordinates. The extracted signals
can then be manually sorted to confirm that they represent cellular Ca\textsuperscript{2+} transients from spatially-defined
GCaMP-expressing cells. This technique is particularly helpful for the analysis of dense populations
and allows the rapid identification of thousands of neurons (Jennings et al., 2015; Ziv et al., 2013), but
has also been used to successfully extract individual cellular signals from relatively sparse neuronal
populations such as AGRP neurons located within the arcuate nucleus (Betley et al., 2015).
Alternatively, constrained nonnegative matrix factorization (CNMF) approaches have been developed
for in vivo Ca\textsuperscript{2+} recording analysis (Zhou et al., 2018) that have proven to be more accurate than
PCA/ICA methods. The main advantage is that CNMF methods have the capability of demixing
spatially-overlapping neurons. The CNMF-E (Constrained Nonnegative Matrix Factorization for
microEndoscopic data) variant has an added model to estimate and account for time-varying
background fluctuations in fluorescence (Aharoni and Hoogland, 2019; Zhou et al., 2018). It is however
important to note that all computational methods also have limitations that can reduce the accuracy of
data interpretation (Resendez et al., 2016), and it is currently unclear which method is best suited for
Ca\textsuperscript{2+} indicator data from epifluorescence deep-brain imaging. For a complete review of Ca\textsuperscript{2+} imaging
data analysis and alternative techniques see (Pnevmatikakis, 2019).

\textbf{c. Spike detection}

Spike inference methods aim to estimate the spike times of a neuron given its isolated fluorescence
trace. After ROIs of each active neuron have been identified, \(\delta F/F\) traces can be extracted then
deconvolved to approximate spiking activity of each segment. This process can be challenging due to
the unknown and often non-linear nature of the indicator dynamics, the presence of measurement noise,
and the relatively low imaging rate of typical recordings (Pnevmatikakis, 2019). While CNMF methods
have been successfully used to infer spikes, it should be emphasized that the signal reported by a
fluorescent indicator is a convolution of the Ca\textsuperscript{2+} transients and the indicator response. Although a good
temporal relationship between neuronal firing and GCaMP signals has been reported for several
neuronal cell types (Chen et al., 2013; Dana et al., 2018), this relationship cannot be taken for granted
and Ca\textsuperscript{2+} transients should not be seen as electrical recordings. In order to help with the interpretation
of in vivo GCaMP fluorescence dynamics in specific neuronal populations, it can be informative to
undertake dual electrical- Ca\textsuperscript{2+} imaging experiments in targeted neurons in vitro before performing in
vivo experiments.
d. **Analysis packages**

To facilitate the management of big data sets as well as data analysis by non-experts, open access analysis packages have become available and are constantly improving. The following three packages have been used successfully by the miniscope community: (1) CaImAn, a computational toolbox for large scale calcium imaging analysis, which includes movie handling, motion correction, source extraction, spike deconvolution and result visualisation (Giovannucci et al., 2019), which is the most up-to-date package; (2) MiniscoPy, a python-based package adapted from CaImAn specifically to analyse miniscope recordings (Zhou et al., 2018); and (3) MiniscopeAnalysis, an updated version of the initial miniscope analysis package developed for the miniscope project. As an alternative to these packages, Lu and colleagues (Lu et al., 2018) have developed a fully-automated algorithm called the miniscope 1-photon imaging pipeline (MIN1PIPE), which contains several stand-alone modules and can handle a wide range of imaging conditions and qualities with minimal parameter tuning, and automatically and accurately isolate spatially-localized neural signals. We have worked with MIN1PIPE and found it efficient and easy to use and adapt to our needs without having to be a specialist in data analysis.

Inscopix clients will benefit from the user-friendly ‘Inscopix Data Processing’ software that performs all steps of the analysis (filter, motion correction, cell registration for longitudinal studies, extraction of the signal) with very limited parameter tuning. We have found it very easy to use and convenient for recordings with good SNR and limited motion artefacts.

e. **Computer/hard disk specifications**

The high-definition data acquired during Ca²⁺ imaging sessions require a significant amount of hard disk space during recording (acquiring 30 minutes of data at the frame rate of 20 Hz with the nVista miniscope requires approximately 110 GB of hard disk space) as well as a substantial amount of space for data storage. This problem should be thought through before starting any recordings. Moreover, in order to perform longitudinal studies, movies from different recording sessions will need to be concatenated into a single large file, and powerful computers with numerous cores will be required for analysis.
7. Readout and physiological consequence of the signals recorded

Once the recordings have been analysed and spatial and temporal data have been extracted, it can still be a challenge to understand the signals. Indeed, within a defined neuronal population, subgroups or individual neurons may be active at different time points, so it is critical to find ways to distinguish the activity we are interested in from the rest.

a. Behavioural/Environmental changes

One of the most notable benefits provided by in vivo imaging of neuronal activity in conscious animals is that it is possible to correlate the signal with contextual changes in environment (e.g. time of the day, temperature, light intensity), behaviour (e.g. environmental exploration, time-locked stimulus presentations), or pharmacological induced alterations of network activity. Indeed, the ability to image cell type-specific activity during naturalistic animal behaviour allows this imaging protocol to be applied to a wide array of experimental manipulations, not only to answer questions that could not be tackled using previous methods for monitoring of neural activity, but to provide clues about the significance of the recorded calcium signals. With the ability to record activity across several days/weeks, each animal can become its own control. But what is perhaps more valuable is that because individual neurons can be registered and therefore studied over several recording sessions, each neuron can become its own control. We have used this experimental design to study the effect of isoflurane on hypothalamic neuronal activity. General anaesthetics, especially isoflurane, are routinely used to induce a sleep-like state, allowing otherwise painful or stressful procedures to be performed, but have been long known to alter hormonal secretion. For example, in rats fed ad libidum, isoflurane increases glycemia, glucagon and GH levels but causes decreases in insulin, ACTH and corticosterone levels (Saha et al., 2005). Similarly, pulsatile LH secretion and LH concentrations are diminished in anaesthetised mouse models (Campos and Herbison, 2014). How exactly isoflurane works in the brain is not fully understood (Campagna et al., 2003), and although its effect on neurons has not been studied in real time in vivo, studies have reported disrupted calcium activity in astrocytes (Thrane et al., 2012) and altered blood flow in the brain (Rungta et al., 2017) following isoflurane anaesthesia. Armed with the ability to image hypothalamic neurons in situ, we have been able to characterise the effect of isoflurane on the Ca²⁺ dynamics of arcuate nucleus neurons. We performed Ca²⁺ imaging in head-restrained mice first in awake conditions, and then for a 25-minute period following anaesthesia induction. We monitored the same neurons in both conditions and found that isoflurane greatly altered the Ca²⁺ activity of the recorded neurons-(Fig3) (Campos and Mollard, unpublished observations).
b. Blood sampling

An important advantage of working with neuroendocrine circuits is that the final readout of the network and its physiological relevance can in many cases be readily assessed through changes in circulating hormone concentrations. The recent advance in ultra-sensitive hormone assays for pituitary hormones (Guillou et al., 2015; Steyn et al., 2011, 2013) have allowed researchers to increase the sampling frequency while decreasing blood volumes needed to get a high-resolution reading of hormonal dynamics. Two main techniques can be effectively combined with in vivo imaging of hypothalamic neurons.

Tail-tip method

The tail-tip technique involves cutting the last 1mm of tail tip and then collecting small 3 to 5μL volumes of blood that can be analysed for hormone concentration using ultra-sensitive assays. The blood flow is encouraged by gently massaging the tail and stops spontaneously when stroking is stopped. Because of the size of the samples this technique results in minimal blood loss and has been shown to be very efficient in characterising LH and GH profiles in mice (Steyn et al., 2011, 2013). To succeed in using this blood sampling method thorough training is necessary, particularly as animals will be either tethered or head-restrained. Animals need to get used to staying in the hand of the experimenter, being restrained by holding the base of the tail, and having their tail massaged. It is important to note that rats are more sensitive than mice with their tail, and we have failed to measure LH surges in female rats after taking frequent blood samples using this technique.

Automated blood sampling

To collect a series of blood samples at high frequency, animals can be implanted with vascular cannula(s) and connected to an automated blood sampling (ABS) system. These systems withdraw small blood samples via the vascular cannula, which are sent to a fraction collector where they are deposited into collection tubes. The advantage of ABS systems is that the blood sampling procedure can be carried out with minimal disturbance to the animal, and the sampling frequency can be specified according to the goal of the study. These experiments are considered stress-free for the animals and have been used extensively to study the regulation of the hypothalamic-pituitary-adrenal axis (Walker et al., 2012; Windle et al., 1998). There are, however, some disadvantages to this technique. First, the animals need to be implanted with a vascular cannula placed in the jugular vein. Because of the difficulty of this surgery, these experiments, although possible in mice (Adams et al., 2015) are better-suited to larger rodents such as rats. Second, the sample volumes are typically greater than those collected using the tail-tip technique. Third, this technique is particularly suited for the measurement of
stable hormones such as corticosterone (Walker et al., 2012). Finally, as animals need to be tethered to perform ABS studies, attention is required to ensure the cable from the miniscope does not become tangled.

- **Conclusions**

Deep-brain imaging techniques have the potential to produce data that were long thought impossible to obtain because of the inaccessibility of the hypothalamic-pituitary system. Overall, being able to study the activity of specific hypothalamic neurons in awake animals and in real time offers an incredible and invaluable opportunity to broaden our understanding of how the brain controls endocrine function. It also gives us the opportunity to study why endocrine functions become disrupted in pathophysiological conditions, and how these changes may lead to the development or even protection from pathological consequences. Furthermore, *in vivo* brain imaging in conscious animals permits longitudinal studies, thus offering the capability to use animals (and even individual neurons) as internal controls prior to a challenge (e.g. stress, pregnancy, circadian perturbation), which is in-line with 3Rs guidelines and compares favourably with approaches involving numerous animals to provide adequate numbers in test and control groups. While these techniques were long thought to be available only to laboratories specialised in optics and engineering, or to laboratories with sufficient funding to purchase ready-made equipment, they are now accessible to the wider endocrine field with the help of open source resources. It is expected that high-quality research and high-impact publications emerging from these methodologies will benefit the scientific and medical communities and open up new research opportunities. While this review has focused on imaging Ca$^{2+}$ dynamics within neurons, many other indicators exist. Of particular interest are Flamindo (Odaka et al., 2014) and R-FlinA (Ohta et al., 2018), which are single-wavelength indicators that permit imaging of 3'5'-cyclic adenosine monophosphate (cAMP) dynamics in living organisms. Likewise iATPSnFRS allows imaging of extracellular and cytosolic adenosine triphosphate (ATP) in astrocytes and neurons (Lobas et al., 2019). Intensive efforts have also resulted in the development of modified G-protein-coupled receptors in which a permuted GFP has been inserted into the third loop in order to monitor various signalling molecules. While this technology currently only works for the detection of small neurotransmitters such as acetylcholine (Jing et al., 2018) and dopamine (Patriarchi et al., 2018), one can hope that these tools will soon be suitable for *in vivo* detection of peptides and neuropeptides.
Acknowledgments: The authors acknowledge Pr. Bradley Lowell for kindly providing TRH-Cre mice. PC and JJW acknowledge financial support from the Medical Research Council (fellowship MR/N008936/1 to JJW) and the Engineering and Physical Sciences Research Council (grant EP/N014391/1 to JJW). PC and PM acknowledge financial support from the Agence Nationale de la Recherche (ANR-15-CE14-0012-01, ANR-18-CE14-0017-01), France-Bioimaging (INBS10-GaL/AR-11/12), Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Université de Montpellier, and Fondation pour la Recherche Médicale (DEQ20150331732).

Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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**Figure Legends:**

*Figure 1: Deep-brain imaging as a powerful tool to understand neuroendocrine functioning.*

Unknows such as the relationship between the activity of specific neurons and the dynamics of peripheral hormonal secretion (A), or the way newly discovered genetic mutations result in phenotypic changes (B) can be elucidated using *in vivo* deep brain imaging (C). Researchers will greatly benefit from real-time visualisation of single neuron calcium activity (D), population calcium activity (E), and will gain insight into the network activity of genetically defined neurons (F).

*Figure 2: *In vivo* calcium imaging of TRH neurons in conscious mice.*

A. TRH-Cre mice were injected in the median eminence with a viral vector (AAV5.CAG.Flex.GCaMP6s.WPRE.SV40). B. Immunofluorescence image showing GCaMP6s expression (green) in TRH neurons (red). C. GRIN lens view of a field of the TRH neurons expressing GCAMP6m. Regions of interest corresponding to individual neurons are indicated by coloured arrows. D. Time-lapse recordings of calcium (GCaMP6s) activity of 8 hypophysiotropic TRH neurons in an adult TRH-Cre male mouse. Scale bars 50 μm

*Figure 3: Isoflurane changes the calcium activity of arcuate nucleus neurons.*

25-minute-long *in vivo* recordings of arcuate nucleus neurons were performed in head-fixed mice, mice were then anaesthetised with isoflurane and the same neurons were recorded for another 25 minutes. B. Time-lapse recordings of calcium (GCaMP6s) activity of 7 arcuate nucleus neurons in an awake adult C57Bl6 male mouse. C. Time-lapse recordings of calcium activity of the same 7 arcuate nucleus neurons in the same anaesthetised adult male mouse.
Table 1: Virus Database for the transfection of hypothalamic neurons.

Brain area abbreviations: MS: medial septum; rPOA: rostral preoptic area; AHA: anterior hypothalamus; ARN: arcuate nucleus; PVN: paraventricular nucleus; LH: lateral hypothalamus; RP3V: rostral periventricular region of the third ventricle; VMH: ventro-medial hypothalamus.

Neuronal identity abbreviations: GnRH: gonadotropin-releasing hormone; GHRH: growth hormone-releasing hormone; CRH: corticotropin-releasing hormone; TRH: thyrotropin-releasing hormone; POMC: Pro-opiomelanocortin; AgRP: Agouti-related peptide; GABA: gamma-aminobutyric acid.

Table 2: Comparison of the characteristics of the Inscopix nVista miniature microscope and UCLA Miniscope.

* The field of view given for the 3rd generation UCLA Miniscope is based on imaging superficial brain structures. The addition of an objective GRIN lens that is required to image deep-brain structures reduces this field of view. To our knowledge, precise field of view measurements for deep brain imaging using this miniscope are not known.

Box 1: Typical step-by-step approach toward in vivo deep-brain calcium imaging.
Table 1: Virus database for the transfection of hypothalamic neurons.

| Serotype | Promoter | Transgene | Host Specie | Brain area | Neuronal Identity | References |
|----------|----------|-----------|-------------|------------|-------------------|------------|
| 9        | EF1a     | ChR2      | mouse, rat  | MS, rPOA, AHA | GnRH             | (Campos and Herbison 2014) and unpublished data |
| 9, 5, 2  | EF1a, CAG| ChR2, GCaMP6s | mouse       | ARN        | GHRH              | Unpublished data |
| 9, 5, 2  | EF1a, CAG| ChR2, GCaMP6s | Mouse, rat  | PVN, LH   | CRH               | (Romanov et al. 2017; Füzesi et al. 2016; Pomrenze et al. 2015) |
| 9, 8, 5, 2| EF1a, CAG, Syn| ChR2, GCaMP6s, GCaMP6m, hM3Dq | Mouse | PVN, | TRH              | (Krashes et al. 2014) and unpublished data |
| 9        | EF1a     | ChR2, GCaMP6s, ArchR, HaloR hM3Dq, hM4Di | mouse       | RP3V, ARN | Kisspeptin       | (Clarkson et al. 2017; Han et al. 2015) |
| 5        | CAG      | hM4Di     | mouse       | ARN        | POMC              | (Atasoy et al. 2012) |
| 1, 8     | Syn      | ChR2, ArchR, GCamp6s, hM4Di | mouse       | ARN        | AgRP              | (Atasoy et al. 2012; Betley, Xu, Fang, et al. 2015; Krashes et al. 2014) |
| 1, DJ,9, 5| Syn, Ef1a, | GCamp6s, ChETA, hM3Dq, eYFP | mouse       | LH, ARN    | GABAergic        | (Jennings et al. 2015; Resendez et al. 2016; Silva et al. 2019) |
| 1        | Syn      | GCamp6s   | mouse       | VMH        | Galanergic       | (Viskaitis et al. 2017) |
| 1        | Syn      | GCamp6s   | mouse       | VMH        | Estrogen receptor 1 | (Remedios et al. 2017) |

Footnotes:
Brain area abbreviations: MS: medial septum; rPOA: rostral preoptic area; AHA: anterior hypothalamus; ARN: arcuate nucleus; PVN: paraventricular nucleus; LH: lateral hypothalamus; RP3V: rostral periventricular region of the third ventricle; VMH: ventro-medial hypothalamus.

Neuronal identity abbreviations: GnRH: gonadotropin-releasing hormone; GHRH: growth hormone-releasing hormone; CRH: corticotropin-releasing hormone; TRH: thyrotropin-releasing hormone; POMC: Pro-opiomelanocortin; AgRP: Agouti-related peptide; GABA: gamma-aminobutyric acid.

Table 2: Comparison of the characteristics of the Inscopix nVista miniature microscope and UCLA Miniscope.

| System                  | UCLA Miniscope-3rd generation | nVista 4th generation |
|-------------------------|-------------------------------|-----------------------|
| Weight (g)              | 3                             | 1.8                   |
| Size L x W x H (mm)     | 16.3 x 13 x 22.5              | 8.8 x 15 x 22         |
| Footprint on scull (Baseplate)(mm²) | 75               | 57.44                 |
| Resolution (pixels)     | 752 x 480                     | 1280 x 800            |
| Field of view (µm)      | 700 x 450 *                   | 650 x 900             |
| Sample-rate (Hz)        | 60                            | 50                    |
| Focusing mechanism      | Manual (300 µm) - Slider      | Electronic (300 µm) - Liquid lens |

Footnote:

* The field of view given for the 3rd generation UCLA Miniscope is based on imaging superficial brain structures. The addition of an objective GRIN lens that is required to image deep-brain structures reduces this field of view. To our knowledge, precise field of view measurements for deep brain imaging using this miniscope are not known.
1. **Preliminary steps:**
- Define which calcium Indicator will be best fitted. AIM for high SNRs.
- Define a strategy to target GECIs to the cells of interest:
  - Viral approach: AAVs (most used) vs LVs (large DNA sequences)
  - Transgenic approach: conditional knock-in or promoter-specific expression

2. **Targeting and testing GECIs *ex vivo***:
- Viral approach: Perform stereotaxic surgeries to determine target coordinates, virus volume and concentration and delay for expression
- Viral and transgenic approaches: histological analysis. Nuclear exclusion of GCaMP? Into target cells?
- *Ex vivo* dual electrical-calcium activity recordings of GCaMP-expressing neurons

3. **In vivo calcium imaging**
- Stereotaxic surgeries: GRIN lens implantation
- Histological and physiological tests after GRIN lens implantation
- Animal handling/habitation
- Head-fixed recordings preferably in a first instance. Better image quality and easier to perform.
- Miniscope building/sourcing
- Chronic long-term *in vivo* calcium activity recordings
- Control for stress: corticosterone assay
- Experimental paradigm: behavioral tests/blood sampling

4. **Calcium activity analysis.**
- Requires a powerful analysis computer
- Analysis packages or step by step approach
- Signal filtering, motion correction, registration, spike interference
- Match calcium activity data with behavioral/hormonal data
Figure 3: