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Review Article **Open Access**

A review of the techniques used in studying brain functions

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Abstract

The study of brain function has advanced through a diverse range of experimental and imaging techniques, each offering unique insights into neural activity, connectivity, and behaviour. This review highlights ten widely used methods: electroencephalography (EEG), positron emission tomography (PET), functional magnetic resonance imaging (fMRI), lesion studies, pharmacological interventions, calcium imaging, optogenetics, chemogenetics, fibre photometry, and in vivo electrophysiology. For each technique, the underlying principles, procedures, and typical applications are outlined. Non-invasive methods such as EEG, PET, and fMRI are invaluable for examining large-scale brain dynamics and diagnosing human neurological disorders. However, they face inherent limitations in spatial or temporal resolutions. Invasive techniques, including calcium imaging, optogenetics, chemogenetics, fibre photometry, and electrophysiology, offer cell-type specificity and real-time monitoring of neural circuits. However, technical complexity and invasiveness limit their translational potential. Lesion and pharmacological studies provide causal evidence of brain–behaviour relationships, though reproducibility and specificity remain challenges. By synthesising the strengths and limitations of these complementary methods, this review emphasises the necessity of integrating traditional and emerging approaches. Such integration provides a more comprehensive framework for understanding neural mechanisms and holds promise for advancing diagnostics and therapeutic strategies in neuroscience and clinical practice.

Keywords
Brain functions, Electroencephalography, Functional magnetic resonance imaging, Non-invasive techniques, Positron emission tomography

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Introduction

A fundamental objective in neuroscience is to elucidate the workings of the brain. Given that neural processes unfold across diverse spatial and temporal dimensions, a singular experimental approach is insufficient to fully encompass the intricacies of cerebral activity. Initial understanding was gained through lesion analyses and pharmacological trials, which established connections between behavioural modifications and damage to specific brain areas or chemical modulation of neural function. Subsequently, the discipline has experienced a surge in sophisticated methodologies, from cutting-edge neuroimaging to molecular and optical techniques, enabling researchers to observe and influence neural circuits with remarkable exactness (Boyden *et al*., 2005; Simpson *et al*., 2024).

Amidst this methodological abundance, every individual technique possesses inherent advantages and drawbacks. Non-invasive modalities such as electroencephalography (EEG), positron emission tomography (PET), and functional magnetic resonance imaging (fMRI) are crucial for exploring the human brain in its natural state. Nevertheless, they sacrifice temporal or spatial precision in favour of accessibility and safety. Invasive procedures such as electrophysiology, calcium imaging, and optogenetics reveal intricate circuit dynamics, but their application primarily remains confined to animal studies. Lesion and pharmacological strategies continue to hold relevance, offering causative insights that augment newer technologies (Jun *et al*., 2017; Peirce *et al*., 2019; Simpson *et al*., 2024).

This review employed a narrative approach to examine ten principal techniques in neurophysiological research. A comprehensive literature search was conducted across major scientific databases, including PubMed, Google Scholar, and Web of Science, focusing on peer-reviewed articles, seminal original papers, and authoritative review articles published over the past two decades. The techniques are categorised into non-invasive and invasive groups for clarity. For each technique, we extracted information on underlying foundational principles, procedural steps, primary applications, and inherent limitations. This structure makes it easier to compare and understand how these tools work together to move neuroscience research forward.

This review focuses on ten major techniques currently used in neurophysiology, dividing them into non-invasive (EEG, PET, and fMRI) and invasive (*in vivo* electrophysiology, optogenetics, chemogenetics, calcium imaging, lesion studies, and pharmacological methods). By outlining their principles, procedures, applications, and limitations and emphasising their complementary nature, we aim to provide a balanced perspective on how these tools collectively advance our understanding of brain function and inform translational neuroscience.

Non-Invasive Techniques

EEG

EEG is a non-invasive technique used to record electrical activity in the brain. By measuring voltage fluctuations resulting from ionic current flows within neurons, it provides important insights into neural dynamics (Ramakrishnan *et al.,* 2024). In the clinical settings, EEG is one of the oldest and most widely used neurophysiological techniques. First recorded by Hans Berger in 1924, EEG involves the measurement of brain waves through electrodes placed on the scalp. Caton was the first to report on the "current in the brain's grey substances onto the open brain". Based on Caton's discovery and those of Beck, Danilevsky, Prawdicz-Neminsky and others, Berger made the first EEG recording on July 6, 1924, during a neurosurgical operation on a 17-year-old boy, performed by the neurosurgeon Nikolai Guleke (Tudor *et al.,* 2005).

Principles of EEG:

EEG is based on the detection of electrical potentials generated by neuronal activity. The primary sources of EEG signals are the postsynaptic potentials produced by pyramidal neurons in the cerebral cortex (Buzsáki *et al.,* 2012). Pyramidal neurons are in the cerebral cortex (layers III and IV), the hippocampus and the amygdala. Only the electrical currents in the radially oriented apical dendrites of the neocortical pyramidal neurons can be sampled (Light *et al.,* 2010).

EEG measures the spatial and temporal summations of thousands to millions of synchronously active pyramidal neurons, mostly from dendrites. These pyramidal neurons form dipoles as their dendrites point to the cortical surface. Dipoles refer to a pair of opposite electrical charges separated by a small distance, hence creating an electric field. When groups of neurons are activated, current flows through their dendrites (negative) and soma, thus forming a dipole (positive pole) which can be recorded through electrodes attached to the scalp. Dipoles determine the strength and shape of EEG signals (Peirce *et al.,* 2019).

The standard EEG setup includes electrodes arranged according to the International 10-20 system. Signals from these electrodes are amplified and filtered to remove artefacts. EEG waveforms are categorised based on their frequency bands: delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), beta (13–30 Hz), and gamma (>30 Hz) (Fig. 1). Each frequency band is associated with specific physiological or cognitive states (Oostenveld *et al.,* 2011). EEG recordings can be continuous or event-related, where brain responses to specific stimuli are measured. These brain waves reflect the activity of large groups of neurons and are critical for understanding various states of consciousness, cognitive functions, and pathophysiological conditions. Advanced techniques such as source localisation, coherence analysis, and time-frequency decomposition enhance the interpretation of EEG data (Peirce *et al.,* 2019).

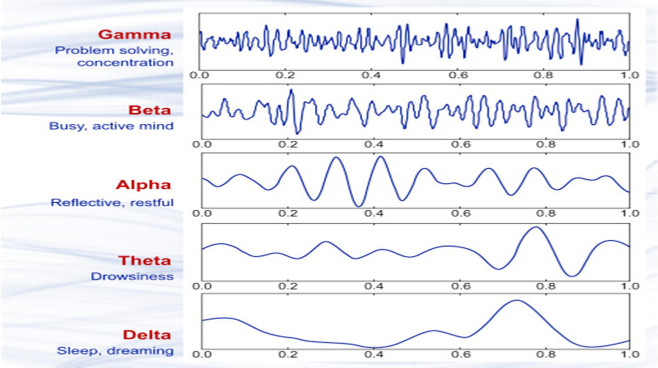

Fig 1: The waveforms of EEG bands (Abhang *et al.*, 2016)

Applications of EEG:

EEG is widely used in clinical diagnostics (Niedermeyer and Lopes da Silva, 2005), particularly in:

- Epilepsy: Detecting and localising seizure activity.
- Sleep disorders: Studying sleep stages and diagnosing conditions like insomnia, narcolepsy, and sleep apnoea.
- Coma and brain death: Assessing brain activity in unconscious patients or after head trauma.
- Neurodevelopmental and psychiatric disorders: Monitoring conditions such as autism, dementia, Attention Deficit Hyperactivity Disorder, and schizophrenia.

- Monitoring brain activity during surgery.

In research, EEG is applied in:

- Cognitive neuroscience: Studying attention, perception, memory, and decision-making.
- Brain-computer interfaces: Enabling direct communication between the brain and external devices.
- Neurofeedback training: Helping individuals learn to regulate their brain activity.

Limitations of EEG:

- Poor spatial resolution: It cannot pinpoint the exact source of brain activity.
- Sensitivity to artefacts: Muscle activity, eye movements, and external electrical noise can contaminate the signals.
- Surface recording: EEG measures cortical activity but has difficulty detecting signals from deeper brain structures.
- Signal interpretation: Requires expertise and careful analysis, as different patterns can have similar appearances.

fMRI

fMRI is an advanced neuroimaging method that detects changes in blood oxygen levels and flow related to neural activity. It enhances traditional MRI by focusing on brain function rather than just structure. The connection between neural activity and blood flow was first established in the late 19th century by Charles Roy and Charles Sherrington, who demonstrated the coupling between brain function and cerebral blood flow (Sandrone *et al.*, 2014). The fundamental basis for fMRI was laid when Linus Pauling and Charles Coryell identified the differing magnetic properties of oxygenated and deoxygenated blood in 1936. Key components of an fMRI setup include the magnet, gradient coil, radiofrequency coil, prism glasses, radio-frequency amplifier, stimulus control computer, spectrometer control computer, button response box, magnetic field amplifiers, video screen, video projector, and headphones (Fig. 3).

Principle of fMRI:

The principle of fMRI involves detecting changes in blood oxygenation in the brain linked to neuronal activity. A localised increase in blood flow that delivers oxygen-rich haemoglobin while lowering oxygen-poor (deoxygenated) haemoglobin levels results from neurons' increased oxygen requirements during activation (Fig. 2). The reduction of deoxygenated haemoglobin increases the MRI signal known as the blood-oxygen-level dependent (BOLD) contrast because it is paramagnetic and distorts the magnetic field. This hemodynamic response peaks when oxygenated blood overcompensates, and it lags neural activity by approximately 4 to 6 sec. fMRI indirectly tracks brain activity with millimetre spatial and second-scale temporal precision by monitoring these BOLD signal fluctuations over time and across brain areas (Huettel *et al.*, 2009). Furthermore, fMRI is a vital tool in neuroscience, psychology, and clinical diagnostics despite issues like noise and poor temporal precision. It enables dynamic, non-invasive viewing of how the brain responds to cognitive tasks, sensory stimuli, or resting conditions.

Unlike X-rays or computed tomography scans, MRI does not involve ionising radiation. Rather than touching the patient, it aligns the hydrogen protons naturally present in the body using a powerful magnetic field produced by coils carrying electric current. This alignment is subsequently perturbed by radiofrequency pulses, and protons produce signals that vary with tissue type and oxygen content as they return to their initial state. These signals are recorded and converted into high-resolution, voxel-based images, which are composed of small units.

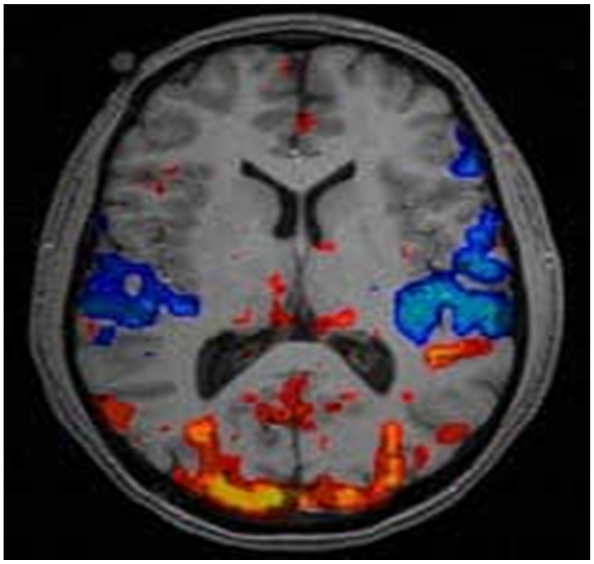

Fig. 2: A voxel image displaying the rate of blood flow. Red represents higher blood flow; Blue represents lower blood flow (OpenLearn, 2022).

Procedure for fMRI:

1. During an fMRI scan, a patient engages in tasks like finger-tapping, reading, or viewing images, which locally boost metabolic activity in the corresponding brain regions. This neural activity causes a vascular response characterised by an initial brief decrease in oxygenation, known as the "initial dip", followed by an overcompensation with increased blood flow and oxygenated haemoglobin (shown in red on the voxels). Since deoxygenated haemoglobin is paramagnetic and distorts the MRI signal, while oxygenated haemoglobin is diamagnetic and enhances the signal, this hemodynamic response called the BOLD contrast peaks approximately 4 to 6 sec after neuronal activation, enabling the detection of task-specific brain activity.
2. Images collected over time enable researchers to monitor signal fluctuations in each voxel and compare them to expected patterns related to the task. This process produces activation maps that reveal brain regions involved. Due to the temporal delay and smooth shape of the hemodynamic response, statistical modelling is necessary to distinguish genuine neural signals from noise.
3. During the resting state, where no task is performed. Instead, low-frequency fluctuations in BOLD signals (<0.1 Hz) across different brain regions are analyzed to infer

functional connectivity and identify networks active at rest (default mode network) or during tasks (task-positive networks) (Khanna *et al.,* 2015).

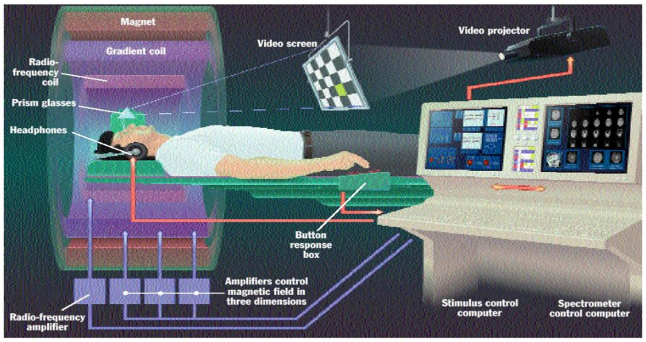

Fig. 3: fMRI Setup (Kesavadas and Thomas, 2008).

Application of fMRI:
Clinical settings

- Mainly employed for pre-surgical brain mapping to locate essential regions responsible for speech, movement, sensation, and cognition (Ciavarro *et al*., 2021).
- It helps surgeons plan procedures to prevent damage to vital brain regions, especially during tumour removal and epilepsy surgeries. Assists in identifying hemispheric dominance for language and memory, which informs surgical choices such as awake craniotomies.
- Helpful for tracking brain recovery following a stroke and assessing the success of medications and behavioural treatments.

Clinical application encounters challenges such as patient movement, tumour influence on blood flow, and the impact of medication on the haemodynamic response (Silva *et al*., 2017).

Neuroscience research

- By tracking blood oxygen levels linked to cerebral function, it provides a non-invasive way to see brain activity. It is also frequently used in studies of neurological disorders, cognition, brain connections, and sensory and motor processing.
- Animal fMRI helps validate human discoveries and understand the brain mechanisms underlying them, but it has practical challenges. It is mostly used on non-human primates.

Limitations of fMRI:

- Baseline challenges: Establishing an exact baseline for comparison during tasks is difficult since the brain is constantly engaged.
- Head motion: Movement during scanning reduces data accuracy by introducing noise and misalignment.
- Limitations of block design: Offers strong statistical power but lacks unpredictability, which could make stimuli predictable and affect organic reactions.
- Event-related design limitations: It offers randomised, adaptable stimuli, but due to smaller signal changes, it has less statistical power.

PET

PET is a functional imaging technique based on detecting the radiation emitted from a radioactive tracer introduced into the body. The historical development of PET dates back to the 1950s, with significant contributions from researchers Gordon Brownell, Charles Burnham, and colleagues at Massachusetts General Hospital (Sweet and Brownell, 1953). They were among the first to demonstrate the use of annihilation radiation as a means for medical imaging. Their pioneering work laid the foundation for medical PET imaging technology, which has since evolved into a crucial tool in both clinical and research settings. PET is a non-invasive technique that provides information on a molecular and cellular level, which makes it particularly valuable for the detection of biochemical changes at an early stage of disease development, which is often before clinical symptoms are evident (Rong *et al.,* 2023).

Principle of PET:
The principle behind PET (Fig. 4) relies on the use of radiopharmaceuticals, radioisotopes attached to biologically active molecules, that undergo beta plus decay. During this decay, a positron (the antimatter counterpart of the electron) is emitted (Vaquero and Kinahan, 2015). When a positron encounters an electron, they annihilate each other, producing two gamma photons emitted in opposite directions. These gamma rays are detected simultaneously by a ring of gamma cameras surrounding the patient, allowing the system to pinpoint the location of the annihilation events within the body and reconstruct detailed three-dimensional images of biological processes at the molecular level (Vaquero and Kinahan, 2015).
The workflow includes:

1. Radiotracer Preparation and Injection: A radiotracer is prepared by incorporating a positron-emitting radionuclide into a biologically active molecule relevant to the target process. The most common radiotracer is fluorodeoxyglucose (FDG), a glucose analogue labelled with fluorine-18. The radiotracer is injected intravenously into the patient, typically through the blood circulation.
2. Tracer uptake and waiting period: After injection, the radiotracer travels through the blood and collects in brain regions based on biochemical or metabolic activity. The average waiting period for FDG is about an hour, which allows the tracer to build up in specific locations.
3. PET scan acquisition: The patient lies inside the PET scanner, which has a ring of gamma-ray detectors for acquisition. Positrons are released when the radiotracer decays by positron emission, and they annihilate with electrons in the surrounding tissue. By using coincidence detection, the scanner's detectors simultaneously identify the pairs of gamma rays that are produced by this annihilation event and released at approximately 180 degrees apart.
4. Localisation of annihilation events: The system determines the origin of the annihilation along a line of response, which is the straight path between the two detectors that detect gamma photons simultaneously. Improvements in timing resolution can enhance the localisation accuracy along this line.

5. Image reconstruction: To produce three-dimensional images that display the distribution of radiotracers, raw coincidence data is processed using sophisticated methods, such as iterative statistical techniques. The metabolic and biochemical activity in the brain tissues is shown in these pictures. Photon scatter, random coincidences, tissue attenuation, and detector sensitivity are all accounted for during reconstruction.

6. Data analysis: Reconstructed images are examined to quantify metabolic or neurochemical parameters important for neuroscience research, such as glucose metabolism or receptor binding patterns.

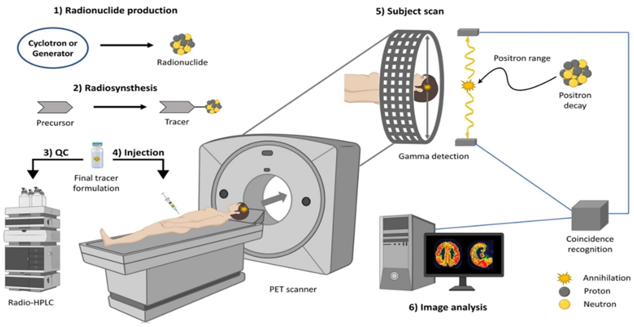

Fig. 4: Illustration of the principle of positron PET (Rong *et al.* (2023).

Application of PET:

In clinical settings

- PET is used in oncology, radiotracers such as ^{18}F-FDG are used for tumour detection, staging, and monitoring treatment responses, highlighting areas of high glucose metabolism typical of cancer cells. Cancer diagnosis, staging, and monitoring (using FDG to detect tumours and metastases)
- PET is used in detecting infection through inflammation imaging.
- PET is used in muscle and bone metabolism imaging
- PET is used in guidance system for image-guided brain lesion surgery.

Neuroscience research

- PET offers molecular-level insights by non-invasively imaging brain metabolism, blood flow, and receptor binding.
- It enables the monitoring of neurochemical changes using radioligands that are selective for receptors and transporters related to dopamine, serotonin, acetylcholine, opioids, and others, thereby improving our understanding of neuropsychiatric and neurological disorders.
- PET is instrumental in early diagnosis and monitoring of diseases like Alzheimer's by imaging amyloid-beta plaques and acetylcholinesterase activity using novel probes.
- Small-animal PET scanners facilitate preclinical studies by allowing repeated measurements over time, which enhances drug development by decreasing the number of animals required and boosting statistical power.
- Advanced PET techniques in neuroscience encompass imaging enzyme activity and neuroreceptor pools, aiding psychiatric research and neuropharmacology (Vaquero and Kinahan, 2015).

Invasive Techniques

In vivo electrophysiology

In vivo electrophysiology encompasses a collection of techniques that capture electrical signals from neurons and neural networks within live, unrestrained animals, frequently during behavioural tasks or sensory input. These methods, which vary from single-cell intracellular recordings to multi-site extracellular probes with high-density configurations, are essential for deciphering how neuronal action potentials, subthreshold membrane activities, and network oscillations contribute to sensory processing, motor control, learning, and cognition (Fig. 5; Buzsáki *et al*., 2012). Progress in microfabricated probe technology, automated intracellular access, and computational spike-sorting software has greatly expanded recording capabilities, permitting large-scale monitoring of neural activity across different brain regions with resolution at the single-neurone level (Jun *et al*., 2017).

Principles of *in vivo* electrophysiology:

Electrophysiology quantifies voltage or current fluctuations arising from the flow of ions across cellular membranes. Intracellular approaches, which include sharp microelectrode and whole-cell patch-clamp configurations, offer a direct readout of membrane potential and synaptic currents, making them exceptionally suited for exploring subthreshold events, ion channel characteristics, and cellular excitability (Noguchi *et al*., 2021).

Extracellular recordings capture the electric fields produced by neuronal currents. The high-frequency elements of these recordings correspond to action potentials, whereas the low-frequency components reflect local field potentials. Recording setups can involve single wires, tetrodes, bundles of microwires, or planar electrode arrays. Contemporary silicon probes, like Neuropixels, allow for the concurrent recording from hundreds to thousands of sites distributed across multiple brain regions, granting exceptional access to neural activity at the population level (Jun *et al*., 2017). Intermediate techniques, such as juxtacellular recordings, strike a balance between spatial resolution and recording stability. Emerging frontiers include the use of nanowire electrodes and hybrid systems that integrate optical imaging with electrophysiological measurements (Luan *et al*., 2023).

An *in vivo* electrophysiology experiment typically involves electrode implantation or insertion, electrical signal acquisition, behavioural control, and data analysis. The choice of recording device—microwire arrays, silicon probes, or neuropixels—determines spatial coverage, signal quality, and stability. Advances in high-density probes incorporate on-probe amplification and multiplexing, reducing noise and cabling complexity (Jun *et al*., 2017).

Intracellular setups require low-noise amplifiers, precision micromanipulators, and often optical targeting. Motion artefacts from heartbeat or respiration are a major challenge; solutions include motion-compensation algorithms, piezo-driven manipulators, and robotic auto-patching systems (Stoy *et al*., 2021).

Data acquisition requires high sampling rates (≥20 kHz per channel for spikes), stable clocking, and specialised software. Data processing pipelines involve filtering, referencing, spike sorting, and statistical analysis. Frameworks such as Probe Interface facilitate probe mapping, metadata handling, and standardisation across laboratories (Garcia *et al*., 2022).

Applications of *in vivo* electrophysiology:

In vivo electrophysiology has been pivotal in mapping sensory receptive fields, decoding motor intentions, and revealing the temporal structure of neural population dynamics. Single-unit and multi-unit recordings elucidate neural coding principles, while intracellular recordings reveal the synaptic and intrinsic mechanisms underlying these codes (Buzsáki, 2004; Noguchi *et al*., 2021). High-density probes permit simultaneous recordings from distributed brain areas, enabling the study of large-scale network interactions during behaviour, learning, and disease (Jun *et al*., 2017; Luan *et al*., 2023).

In vivo electrophysiology aims to:

- Monitor neuronal activity at single-cell or network levels.
- Correlate neural signals with behaviour, sensory inputs, or motor outputs.
- Explore disease mechanisms in models of epilepsy, Parkinson's disease, chronic pain, etc.
- Assess pharmacological effects of drugs on neuronal excitability.
- Investigate neural plasticity, coding, and oscillatory patterns during tasks

Advantages and limitations of *in vivo* electrophysiology:

The primary strength of *in vivo* electrophysiology lies in its unmatched temporal resolution (sub-millisecond) and ability to directly measure neural activity. Extracellular techniques can scale to large neuronal populations, while intracellular methods offer detailed mechanistic insight. Moreover, technological advancements have improved data throughput, reproducibility, and long-term stability (Garcia *et al*., 2022).

However, extracellular recordings provide indirect measurements of membrane potential and rely on spike sorting, which may be imperfect. Chronic implants may provoke immune responses, causing recording instability over time. Intracellular recordings are technically demanding, sensitive to motion, and low-throughput despite automation improvements (Noguchi *et al*., 2021; Stoy *et al*., 2021).

Fibre photometry

Fibre photometry has swiftly become an essential tool for exploring the connections between brain activity and behaviour in living organisms. Originally developed to track calcium fluctuations using genetically encoded calcium indicators (GECIs) such as genetically encoded calcium indicator (GCAMP), the technique now encompasses a broad array of biosensors for detecting neurotransmitters, neuromodulators, intracellular signals, and other physiological variables (Simpson *et al*., 2024). This expansion has been driven by advances in genetically encoded sensor design, viral delivery systems, and compact optical hardware (Kielbinski and Bernacka, 2024). Unlike traditional electrophysiology, fibre photometry enables molecular and anatomically specific recordings over extended time periods in freely behaving animals, thereby providing a powerful link between molecular signalling and behaviour.

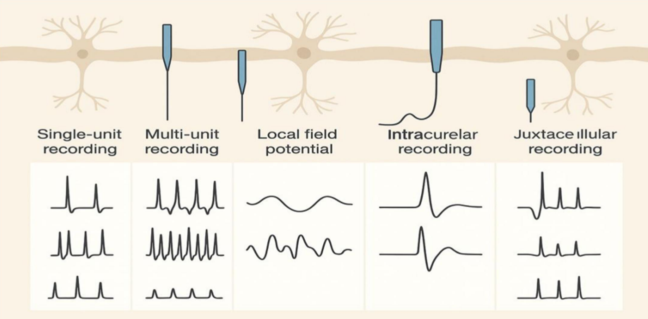

Fig. 5: Types of signal recordings (Buzsáki *et al*., 2012).

Principles of fibre photometry:

Fibre photometry constitutes an optical recording approach where excitation light is transmitted through an implanted optical fibre to fluorescent biosensors located within targeted cells or circuits (Simpson *et al*., 2024). When these biosensors bind to a particular analyte or experience a conformational shift (for example, in reaction to calcium entry), their fluorescence emission intensity changes. The same optical pathway collects the returning fluorescent light, which is subsequently isolated from the excitation beam via dichroic mirrors and optical filters before being registered by a photodetector. The outcome is a bulk fluorescence measurement originating from the tissue surrounding the fibre tip, generally within a radius of 50 to 400 µm (Simpson *et al*., 2024; Kielbinski and Bernacka, 2024).

Most biosensors used in fibre photometry are genetically encoded and composed of two main domains: a sensing domain (responsive to a ligand, ion, voltage change, or other physical parameter) and a fluorescent reporter domain (often a circularly permuted fluorescent protein such as cpGFP). These sensors can be broadly categorised into classes based on their molecular targets:

- GECIs: e.g., GCaMP family (Chen *et al*., 2013).
- Genetically encoded voltage indicators (GEVIs): e.g., accelerated sensor of action potentials family (St-Pierre *et al*., 2014)..
- G-protein-coupled receptors (GPCR)-based neurotransmitter sensors: e.g., dLight family for dopamine (Patriarchi *et al*., 2018), G protein-coupled receptor Activation-based (GRAB) sensors for acetylcholine, serotonin, norepinephrine (Sun *et al*., 2020).
- Periplasmic binding protein (PBP)-based sensors: e.g., intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) for glutamate (Marvin *et al*., 2013).

Intracellular signalling sensors: e.g., cyclic AMP (cAMP) difference detector *in situ* for CAMP (Tewson *et al*., 2016), A-kinase reporter for protein kinase A (activity (Zhang *et al*., 2001).

- pH and redox sensors: e.g., pH-sensing fluorescent probes (Miesenböck *et al*., 1998), The reduction-oxidation sensitive green fluorescent protein (Dooley *et al*., 2004).

Data from fibre photometry are typically processed to express relative fluorescence changes (ΔF/F), controlling for photobleaching, movement artefacts, and baseline drifts. While absolute analyte concentrations cannot be easily inferred due to tissue scattering and variable sensor expression, relative kinetics and event-related changes are highly reliable when supported by appropriate controls.

Experimental setup for fibre photometry:

A typical experiment includes biosensor delivery, optical fibre implantation, data acquisition, and signal processing. Viral vectors, commonly adeno-associated viruses, are used to deliver biosensors under specific promoters to target cell populations. Optical fibres (flat-cut or tapered) are implanted stereotaxically into the region of interest.

The photometry hardware consists of light sources (light-emitting diodes or lasers), dichroic mirrors and filters to separate excitation and emission light, and photodetectors or cameras to detect fluorescence. Multi-channel systems allow recording from different fluorophores or the inclusion of an isosbestic control wavelength for artefact correction (Simpson *et al*., 2024). Signal acquisition software (e.g., photometry modular analysis tool and guided photometry analysis in python) supports preprocessing steps such as filtering, bleaching correction, and normalisation.

Advanced hardware developments include spectrally resolved photometry for improved multiplexing, depth-resolved photometry using tapered fibres, and fluorescence lifetime photometry for absolute quantification (Kielbinski and Bernacka, 2024).

Applications of fibre photometry:

Fibre photometry is widely used to study neurotransmitter release, neuronal activity, and intracellular signalling during behavior (Fig. 6). For example, dopamine sensors like dLight have revealed sub-second fluctuations in striatal dopamine during reward-related tasks (Patriarchi *et al*., 2018). Glutamate sensors like iGluSnFR have been applied to map synaptic activity during learning and memory paradigms (Marvin *et al*., 2013). The method has been combined with optogenetics to link causal manipulations to neuromodulator dynamics (Simpson *et al*., 2024).

Applications extend to astrocytic calcium imaging (Tan *et al*., 2023), circadian rhythm gene expression monitoring (Mei *et al*., 2018), and probing neurovascular interactions. Its adaptability to freely moving preparations makes it ideal for linking molecular events to naturalistic behaviours.

Advantages and limitations of fibre photometry:

Fibre photometry is less invasive than imaging techniques like two-photon microscopy and allows chronic recordings with high temporal resolution. It detects molecules inaccessible to electrochemical probes and enables multiplexing for complex circuit analyses.

However, limitations include the inability to resolve single cells, difficulty in absolute quantification, and susceptibility to confounding signals from hemodynamic or pH changes. Signal interpretation requires careful use of controls such as isosbestic excitation, reference fluorophores, or ligand-binding-deficient sensor mutants (Simpson *et al*., 2024). Signal-to-noise ratio: The signal can be noisy, especially in long-term recordings (Legaria *et al*., 2022).

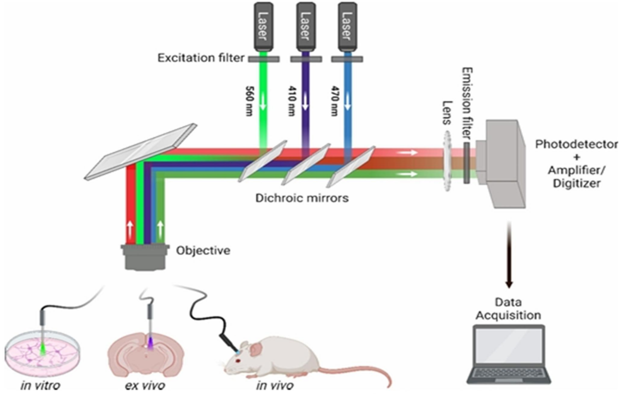

Fig. 6: An illustration showing the principle of action for a conventional photometry system (CPS). Kielbinski and Bernacka (2024)

Optogenetics

Comprehension of the brain function can be advantageous for the therapy of neurodegenerative diseases. The brain consists of various types of neuron sets, which are organised in three-dimensional complex networks and form neural circuits underlying different behaviours. The circuits act based on the patterns that encode the brain functions. Recognition of the neural patterns requires methods to manipulate the neurons. Electrical stimulation may be the most common method. However, it has drawbacks, including failure to identify specific neurons in experiments. As an alternative, optical stimulation is a new method that acts in combination with genetic approaches. The novel, optogenetic technology makes it feasible to manipulate either the specific cell types or the neural circuits. This technique is associated with minimal tissue damage as well as side effects.

In 2005, optogenetics was born and appeared in the public (Boyden *et al.,* 2005). The seminar paper reported an effective method to excite neurons in rats using the opsin channelrhodopsin (derived from green algae). In the following years, many research groups have gone on to expand optogenetics' possibilities. Therefore, optogenetics has made many achievements in the field of neurobiology. This includes exploring unknown neurone functions, discovering neural circuits, and treating neurological diseases.

Optogenetics is a neuromodulation approach that controls the neural activity using light (Fig. 7). This method functions on the basis of the bioengineered light-sensitive proteins. It can optionally stimulate or silence particular cell types and neuronal circuits with millisecond temporal accuracy. This method allows for greater temporal resolution when analysing specific neural circuit operations in different diseases (Yizhar *et al.*, 2011).

Principle of optogenetics

Viral vector delivery: Modified adenoviruses or adeno-associated viruses deliver opsin genes to target neurons.

Opsin types: Microbial-derived proteins serve as light-gated ion channels/pumps (Fig. 9):

- Channelrhodopsin-2 (ChR2): It is excitatory and activated by blue light (470 nm).
- Halorhodopsin (NpHR): It is inhibitory and activated by yellow light (589 nm).
- Retinal cofactor requirement: All opsins require retinal (vitamin A derivative) for light sensitivity.
- Optical activation: Fibre optics or implanted light-emitting diodes deliver millisecond-precision light pulses to target brain regions.
- Ion flux mechanism: Excitation/Inhibition.
- Behavioural monitoring: by turning on or off the activity of each cell type, to determine the role that each cell type plays in different behaviours.

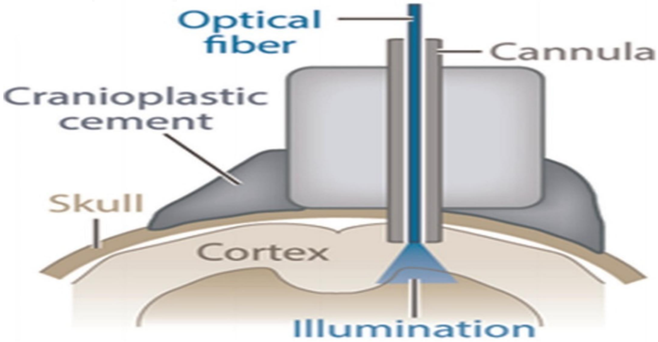

Fig. 7: An optical fibre implanted into the brain for light delivery (Hadi *et al.,* 2017)

Retinal is one of the many forms of vitamin A, and all-trans-retinal is also an essential component of microbial opsins. In these molecules, light transforms 11-cis-retinal to all-trans-retinal, which cycles back to 11-cis-retinal with the dark condition. 11-cis-Retinal can covalently compound with the protein ChR2 and then alter to all-trans-retinal by a blue photon with a wavelength of 470 nm (Fig. 8).

ChR2 is an ion channel that is activated in the presence of blue light (~470 nm) and allows the influx of cations (Na^+). The result is cell depolarisation that eventually leads to neuronal firing.

Volvox channelrhodopsin-1 (VChR1), like ChR2, is an ion channel. VChR1, however, is activated by yellow light (~589 nm) to allow influx of Na^+ and elicit action potential.

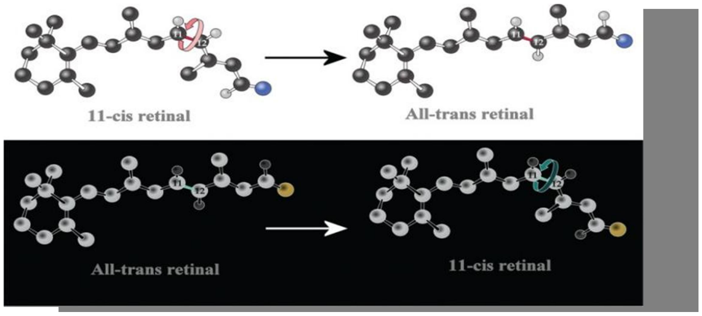

Fig. 8: Isomerisation of 11-cis-retinal by illustration (Hadi *et al*., 2017)

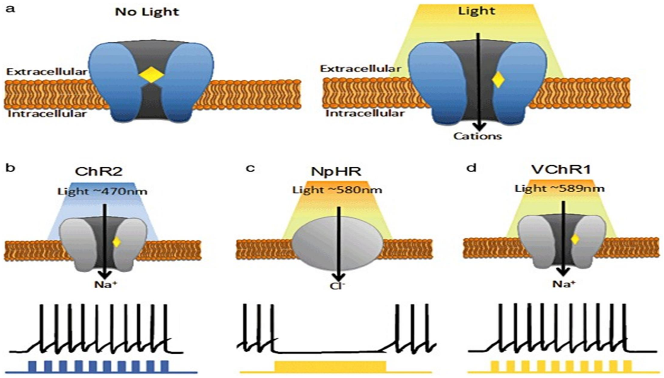

Fig. 9: Principles of optogenetic tools (Zhen *et al.,* 2011).

Halorhodopsin obtained from the halophilic bacterium Natronobacterium pharaonis (NpHR) is the principal rhodopsin that inhibits the neuronal activity. NpHR acts as a chloride pump. It is sensitive to yellow light with a wavelength of 580 nm; therefore, one chloride ion enters into the cell through its membrane per one yellow photon that the nerve cell receives. This process hyperpolarises the neuron (Nagel *et al.,* 2003).

Applications of optogenetics:

- Neural circuit mapping and connectivity: Exploring how various brain regions communicate (Chuhma, 2021).
- Studies of the visual cortex showed different inhibition patterns: activating parvalbumin interneurons led to divisive inhibition, whereas somatostatin interneurons caused subtractive inhibition in pyramidal neurons (Wilson *et al*., 2012). Projections from the prefrontal cortex to the dorsal raphe nucleus enhanced motivation-related behaviours in rats, while those to the lateral habenula reduced them (Warden *et al*., 2012).
- Behaviour and cognitive function analysis: associating particular neuronal groups with specific behaviours (Adamantidis *et al*., 2007).
- Decision-making: Inactivating neurons in the nucleus accumbens of rats changed how rewards are processed and led to more complex behaviours (Aquili *et al*., 2014). Memory formation: Researchers restored spatial memory in Alzheimer's mouse models by repairing synaptic connections within the entorhinal-hippocampal circuits (Yang *et al*., 2019).

- Examining disease mechanisms: Optogenetics has provided crucial information about neurological problems such as epilepsy (Bentley *et al*., 2013), Parkinson's disease (Lee *et al*., 2018), and mental illnesses (Touriño *et al*., 2013). Therapeutic Development: Using ChrimsonR opsin to partially restore vision in individuals with retinitis pigmentosa (Sahel *et al*., 2021).

Advantages of optogenetics:

- Optical techniques are more useful than pharmaceutical and electrical techniques because of their higher speed, accuracy, and less tissue damage.
- Achieving millisecond accuracy to replicate natural neural firing patterns. Bidirectional control (activation and inhibition) within a single experiment. Cell-type specificity through genetic targeting
- Multiplexed manipulation using opsins with different light sensitivities

Limitations of optogenetics:

In order to reach deep brain regions, an intrusive implantation is necessary.

- Extended exposure to light can heat or harm tissue.
- Because of ethical and technical issues, translation to humans is still in its infancy.
- Opsin expression in unwanted cell types or brain regions could result from viral vector leakage.
- Prolonged usage of chloride pumps (eNpHR) disrupts GABAergic signalling by affecting chloride ion gradients.

Chemogenetics

During the last decade, there has been a revolution in neuroscience techniques that have resulted in increasingly precise methods to manipulate neural systems in awake, behaving animals. Understanding the relationship between brain function and behaviour is critical for the advancement of neuroscience research and targeted medication development. Chemogenetics refers to the technique that allows for the reversible remote control of cell populations and neural circuitry *via* systemic injection or micro-infusion of an activating ligand (Alexander *et al.,* 2009; Armbruster *et al.,* 2007).

Chemogenetics, also called designer receptors exclusively activated by designer drugs (DREADDs), emerged as a powerful technique to remotely and selectively control neuronal activity through engineered receptors responsive to otherwise inert small molecules (Roth, 2016). This approach has revolutionised neuroscience by enabling the non-invasive, reversible, and cell-type-specific control of brain circuits in living animals, thereby bridging molecular neuroscience with behaviour.

Principles of Chemogenetics:

Chemogenetics is based on the genetic engineering of GPCRs so that they are unresponsive to endogenous ligands but can be selectively activated or inhibited by synthetic, otherwise pharmacologically inert compounds (Armbruster *et al.,* 2007). These engineered receptors, when expressed in specific neurons, allow the experimenter to precisely control neuronal excitability or signalling pathways (Fig. 10).

The most widely used chemogenetic tools are DREADDs, derived from muscarinic acetylcholine receptors that were modified to no longer respond to acetylcholine but instead to a synthetic ligand such as clozapine-N-oxide (CNO), and activation can lead to neuronal excitation, inhibition, or modulation of intracellular signalling (Roth, 2016).

Procedures for chemogenetics:

1. Genetic targeting: Under selective promoters, genes encoding DREADDs are introduced into particular brain regions or cell types via viral vectors (such as adeno-associated viruses) or transgenic techniques (Alexander *et al*., 2009).
2. Expression of engineered receptors: The modified GPCRs are visible on the surface of the target neurons. membranes.
3. Administering the designer ligand: The synthetic ligand, typically CNO or low-dose clozapine, is delivered systemically via intraperitoneal injection or oral gavage (Fig.11).
4. Selective activation or inhibition: Only the engineered receptors in genetically targeted neurons. respond to the ligand, leading to modulation of it, thereby modulating neuronal excitability.

Examples:

- Modified human muscarinic acetylcholine receptor M3 (hM3Dq) DREADD: couples with Gq proteins, raises intracellular Ca^{2+} levels, and boosts excitability.
- Human muscarinic receptor subtype 4, DREADD, inhibitory (hM4Di) DREADD: couples to Gi proteins, decreases cAMP levels, activates G protein-coupled inwardly rectifying potassium channels, and suppresses neuronal firing.
- Kappa-opioid receptor (KOR) DREADD: activated by salvinorin B, it quickly induces inhibition (Vardy *et al*., 2015).
- Functional outcome: Changes in the activity of a specific neuronal circuit result in observable alterations in behaviour, cognition, or physiology.

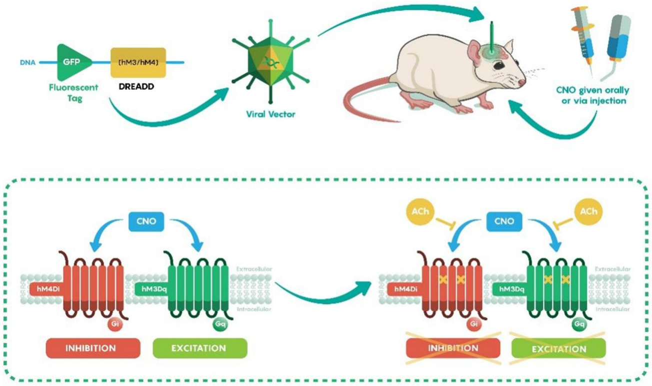

Fig. 10: Schematic overview of how different variants of DREADDs (hM3Dq and hM4Di) can be used to activate and also inhibit groups of neurons using CNO. The figure also shows acetylcholine's inhibitory effects on CNO (Ju, 2023).

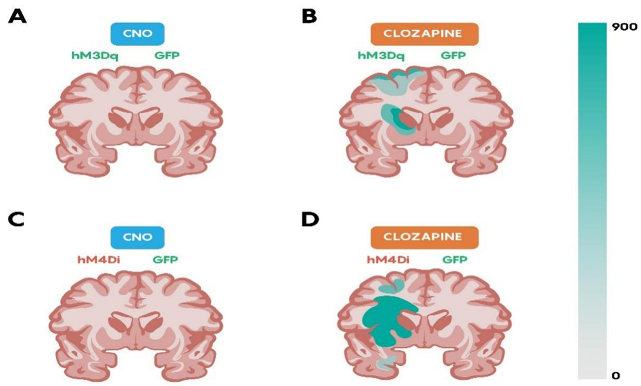

Fig. 11: Showing the lack of relative binding of the ligand CNO (A and C) to the DREADDs versus the metabolite clozapine, which shows significant binding to the same DREADDs (B and D) as indicated by the darker intensity stains (Adapted from Gomez *et al.,* 2017).

Applications of chemogenetics in neuroscience:
Chemogenetics has seen extensive use in both fundamental and applied neuroscience research. Chemogenetics has been widely applied in both basic and translational neuroscience research:
Circuit dissection: Mapping functional contributions of specific neuronal populations to learning, memory, emotion, or motor control (Krashes *et al.,* 2011). Circuit dissection: Identifying how specific neuronal populations contribute to learning, memory, emotion, or motor control (Krashes *et al*., 2011).
Behavioural neuroscience: investigating how reward, addiction, anxiety, and depression are influenced by dopaminergic, serotonergic, and glutamatergic pathways (Timothy *et al*., 2020; Urban *et al*., 2016).
Neurodevelopment and plasticity: Examining the effects of focused neuronal activation on developmental mechanisms, crucial periods, and synaptic remodeling (Pati *et al*., 2020).
Disease modelling: Mimicking or rescuing neural dysfunction in models of Parkinson's disease, epilepsy, schizophrenia, and Alzheimer's disease (Mahler *et al.,* 2019).
Therapeutic exploration: Potential use of targeted neuromodulation for psychiatric and neurological disorders, providing a non-invasive alternative to deep brain stimulation.

Advantages and limitations of chemogenetics:
Due to the ability to selectively modulate certain neuronal populations through genetic targeting, chemogenetics has a high degree of specificity. Since ligands can be administered systemically without the need for implants or light delivery, this also provides a non-invasive mode of control. The method provides fine temporal control and is both reversible and titratable. Its effects depend on the dosage and the presence of the ligand. Behavioural studies and chemogenetics work well together because they allow for the study of free-moving, tether-free animals. Exciting, modulatory, and inhibitory DREADDs may be used to manipulate circuits in a variety of ways (Wang *et al*., 2011; Roth, 2016).

Like other techniques, this method has limitations. For example, ligand concerns such as CNO, widely used, are converted into clozapine *in vivo*, causing off-target effects (Gomez *et al*., 2017). Chemogenetics works on a time-scale of minutes, which is slower than optogenetics with millisecond precision. Viral transduction efficiency can vary, and off-target expression may occur. It is also dose-dependent, and results may be difficult to interpret if it is overactivated or underactivated. Finally, issues with receptor delivery and ligand safety restrict clinical application.

Calcium imaging technique

Comprehending brain function necessitates instruments capable of measuring neuronal activity with both high spatial and temporal fidelity. Calcium imaging stands out as one such widely adopted technique in neuroscience, allowing for the visualisation and measurement of intracellular calcium (Ca^{2+}) dynamics, which serve as an indirect indicator of neuronal activation. So many neuronal processes, including synaptic communication, plasticity, and membrane excitability, involve calcium ions (Grienberger and Konnerth, 2012). This is because action potentials and synaptic events are typically accompanied by transient rises in intracellular Ca^{2+}. Hence, calcium imaging offers a minimally invasive approach to observe activity across extensive neuronal populations in real time. making it crucial for connecting cellular and network-level activity to cognitive processes, behaviour, and disease states.

Principles of calcium imaging technique:
The foundational concept of calcium imaging rests on the observation that neuronal excitation triggers an inflow of Ca^{2+} ions into the cytoplasm, occurring either through voltage-gated calcium channels during action potentials or via receptor-operated pathways. By employing fluorescent calcium indicators, either synthetic chemical dyes or GECIs (Fig. 12 and 13), these shifts in Ca^{2+} concentration can be captured optically (Chen *et al.,* 2013). Upon binding Ca^{2+}, these indicators undergo structural changes that modify their fluorescence intensity or spectral properties.

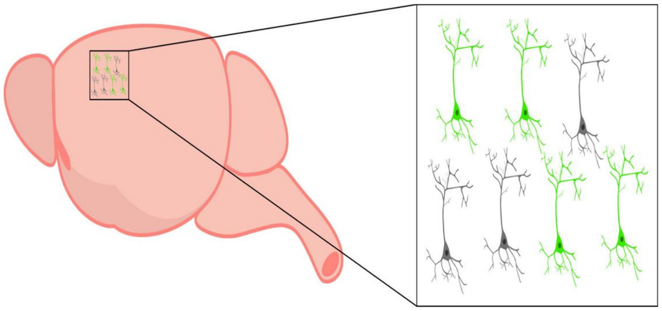

Fig. 12: GECIs, such as GCaMP, are expressed in specific cells (in green). Changes in activity are only detected in cells expressing the GECI (Dana *et al.,* 2014).

The emitted fluorescence is recorded using advanced microscopy techniques such as confocal microscopy, two-photon microscopy, or wide-field fluorescence imaging. The fluorescence intensity is proportional to intracellular Ca^{2+} concentration, thereby serving as a surrogate for neuronal activity.

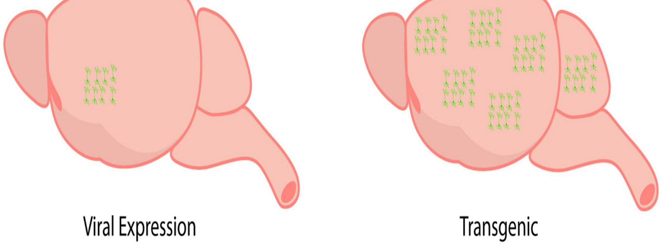

Fig. 13: GECIs can be expressed through viral expression (on the left), and this limits expression to a specific cell type and brain region. In comparison, transgenic expression (on the right) is widespread throughout the brain (Resendez *et al*. 2016).

The experimental workflow includes:
1. Introduction of calcium indicators: The first step is getting the calcium indicators (chemical dyes (e.g., Fura-2, Fluo-4, Oregon Green BAPTA)), which are then loaded into neurons via incubation, microinjection, or patch-clamp loading. In recent times, GECIs such as GCaMPs are introduced using viral vectors or transgenic animal models, allowing cell-type specificity and chronic imaging. This also allows one to pick exactly which neurone types like light.
2. Calcium binding and fluorescence change: Once the indicators are in place, calcium ions roll in during an action potential and bind. Indicators contain Ca^{2+}-binding domains (like calmodulin or BAPTA analogues). Upon binding to Ca^{2+}, these domains change their fluorescence properties (intensity, excitation/emission spectra, or Förster resonance energy transfer—FRET). With FRET-based sensors, energy starts jumping between molecules.
3. Imaging and recording: This next step helps to actually see all that is happening in the previous step. Two-photon microscopes are the gold standard when working with deep tissues, enabling the experimenter to watch neurons fire as the animals run around. Fluorescence is detected by photomultiplier tubes, charge-coupled device cameras, or high-speed detectors. Imaging can be targeted at single neurons, dendrites, or entire neural populations depending on the research question and focus.
4. Signal Processing: The raw fluorescence data or traces ($\Delta F/F$) are processed to reflect relative Ca^{2+} concentration changes, corresponding to neuronal activity such as action potentials, bursts, or network oscillations.

Applications of calcium imaging technique in neuroscience:

- Neural circuit dynamics: Calcium Imaging allows visualisation of population-level activity in cortical and subcortical circuits during sensory processing, decision-making, and motor control (Peron *et al.,* 2015).
- Synaptic plasticity: Calcium signals at dendritic spines are crucial for studying long-term potentiation (LTP) and depression (LTD), which underlie learning and memory (Nevian and Sakmann*,* 2006).
- *In vivo* imaging: Two-photon calcium imaging in live animals enables monitoring of neuronal activity during natural behaviour, revealing real-time brain-behaviour relationships.
- Disease models: Altered calcium signalling is implicated in disorders such as epilepsy, Alzheimer's disease, and Parkinson's disease. Calcium imaging helps uncover pathophysiological mechanisms (Busche and Konnerth, 2015).
- Drug screening and neuropharmacology: Calcium dynamics serve as functional readouts for evaluating the effects of drugs on neuronal excitability and synaptic function (Seshadri *et al.,* 2020).

Advantages and limitations of calcium imaging technique: Calcium signalling has high spatial resolution, allowing imaging from single spines to large neuronal populations. With GECIs, researchers can target specific neurone classes or circuits, and it is relatively non-invasive compared to electrophysiology. It also allows chronic imaging, that is, allowing longitudinal studies over days to months in the same animal. Additionally, simultaneous monitoring of hundreds to thousands of neurons is possible with calcium signalling techniques. Two-photon *in vivo* imaging permits real-time correlation between brain activity and animal behaviour.

The limitations are that calcium signals reflect neuronal activity indirectly, they lag behind action potentials and have slower kinetics (Wei *et al.,* 2020). Fluorescence intensity does not always scale linearly with Ca^{2+} concentration, complicating quantitative interpretation. Prolonged imaging may damage tissue or reduce signal strength (phototoxicity and bleaching). Optical imaging is limited in penetration depth, though two-photon microscopy improves this compared to wide-field methods. Also, viral delivery of GECIs may vary in efficiency and expression levels. Calcium transients cannot capture very fast spiking activity compared to direct electrophysiological recordings (Wei *et al.,* 2020).

Lesion and pharmacological methods

Lesion studies

Lesion technique in neuroscience involves studying individuals or animals with brain damage to understand how specific brain regions contribute to cognition and behaviour (Vaidya *et al*., 2019). This method enables researchers to observe the effects of damage to delicate brain areas, moving beyond simple correlations to establish causal links between brain regions and mental functions, as deficits following a lesion show the necessity of that area (Lavond and Steinmetz, 2003). Despite advances in neuroimaging and neural manipulation, lesion techniques remain crucial because they uniquely demonstrate the necessity of a brain region for certain functions. Originating in the 19th century with scientists like Paul Broca and Carl Wernicke, who identified language-related brain areas through studying patients with speech impairments, these early findings laid the groundwork for localising brain functions based on behavioural effects of damage. Over time, the technique has advanced to include more precise methods, such as voxel-based lesion-symptom mapping, which allows detailed correlations between lesion locations and specific cognitive deficits.

The basic principle behind the lesion technique is simple and straightforward. Damage to a particular brain area and the resulting change or loss in a cognitive or behavioural function suggest that the damaged region is responsible for that function (McCurdy *et al*., 2022). Lesion studies, when compared to other correlational neuroimaging like fMRI or PET, show you direct causal evidence of brain-behaviour links. These lesions may occur naturally, such as from stroke or injury, and researchers explore from there. However, it can be induced experimentally in animal models to test specific hypotheses. More recently, researchers also combine lesion damage with other functional imaging methods to watch how the rest of the network reorganises itself around the damage.

Procedure for lesion technique:

1. Identify the region of the brain to study by considering existing knowledge, hypotheses, or observable behaviours that require explanation.
2. Induce or Identify a Lesion: In human studies, lesions often occur naturally due to stroke, injury, or neurosurgical treatment. In animal studies, lesions can be experimentally induced using surgical or other methods (Table 1) that target the desired brain area, enabling greater precision and controlled timing.
3. Pre-lesion assessment (if feasible): Pre-lesion assessment may not be feasible in all cases, but in certain cases, with animal models or planned neurosurgery, there is a need to do a baseline cognitive or behavioural performance before lesion induction to enable within-subject comparisons.
4. Post-lesion behavioural and cognitive testing: Subjects, whether human or animal, are subjected to various cognitive, sensory, or behavioural assessments following the lesion to identify any resulting changes or deficits in function.
5. Control groups: Appropriate controls, such as healthy people, subjects with lesions outside the target area, or animals undergoing sham surgery, are compared in order to isolate lesion effects.
6. Imaging and lesion verification: To ensure anatomical accuracy, brain imaging methods such as MRI or CT scans are used to confirm the location, size, and extent of lesions.
7. Data analysis and correlation: In order to establish spatially precise connections between damage and impairments, behavioural changes are examined in relation to lesion sites, frequently utilising techniques like voxel-based lesion-symptom mapping.
8. Interpretation: By associating specific deficits with particular lesions, researchers confirm the causal roles of brain regions in certain cognitive or behavioural functions.

Types of lesion induction and methods:

There are several methods for lesion procedures. Most human research uses naturally existing lesions from neurosurgical procedures, stroke, trauma, or malignancies (McCurdy *et al*., 2022). Researchers can infer about the affected brain regions thanks to meticulous participant selection and anatomical mapping, even when these lesions are not controlled experimentally and can vary in size, location, and aetiology. However, by employing the techniques described below, research involving non-human primates and other animals can incorporate induced lesions with significantly greater accuracy (Table 1).

Table 1: Methods, applications and mechanisms of different types of lesions

S/N	Method	Application	Mechanism
1	Natural lesions	Humans (stroke, trauma, tumors)	Study of pre-existing damage; anatomical mapping via MRI (McCurdy *et al.,* 2022).
2	Surgical ablation	Animal models (non-human primates)	Physical removal of tissue; high anatomical precision (Vaidya *et al.,* 2019).
3	Chemical lesions	Animal models	Neurotoxins e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) for dopamine neurons in Parkinson's models) (Fischer *et al.* 1995).
4	Reversible techniques	Acute experiments	Local anesthetics or cooling to temporarily inactivate regions (Vaidya *et al.,* 2019).
5	Genetic lesioning	Molecular studies	Gene manipulation to disrupt neuronal function (Vaidya *et al.,* 2019).

Application of lesion technique

Clinical settings

- Targeted lesions are used to treat neurological disorders such as essential tremor and Parkinson's disease. They help identify specific brain regions responsible for symptoms, which guides therapy. Additionally, imaging these lesions assists in diagnosing and predicting outcomes in stroke and brain injury (Ramin *et al*., 2003).

Neuroscience research

- Lesion studies reveal the causal links between different parts of the brain and how they work (Karnath et al., 2019).
- In controlled environments, the induction of lesions in the animals' brains allows for a thorough investigation of neurological networks, brain plasticity, recovery mechanisms, and behavioural impacts.

- When paired with advanced imaging techniques, lesion mapping enhances comprehension of brain function and plasticity (Zamzam *et al*., 2025).

Limitations of lesion technique:

- Acute dysfunction followed by plasticity: Immediately after a lesion, there is a period of severe dysfunction, but over time surviving neural circuits often reorganise and adapt, which can mask or alter the deficits originally caused by the lesion.
- Plasticity masks deficits: Neural plasticity complicates distinguishing between the direct effects of the lesion and compensatory changes developed during recovery.
- Permanent and Temporary Lesions Reveal Different Functions: Permanent lesions may show what brain functions can ultimately proceed without a given region due to reorganisation, while temporary inactivation reveals the immediate, uncompensated role of the area (Vaidya *et al.,* 2019).
- Variability in expression of deficits: Due to recovery and compensation, the behavioural and cognitive impairments may vary over time and between subjects, limiting the consistency of findings (Vaidya *et al.,* 2019).

Pharmacological studies

For decades, particularly in neuroscience, the use of pharmacological approaches has been fundamental, enabling researchers to study the key functions of neurotransmitters, receptors, and signalling cascades as they relate to brain processes and behaviours. Through the application of agonists, antagonists, and modulatory compounds, scientists can adjust neural function and deduce causal relationships between particular neurochemical systems and cognitive or behavioural outcomes. Despite the advent of sophisticated imaging and genetic techniques, pharmacological strategies remain extensively used because they effectively connect molecular, cellular, and systems-level neuroscience (Millan and Bales, 2013).

Principles of pharmacological studies:

The basic idea behind pharmacological methods is to determine what a neurotransmitter or receptor does using drugs as tools or vehicles. These drugs work at various levels depending on the research. Some attach to neurotransmitter receptors (acting as agonists), while others may block the site (antagonists) or modulate ion channel function. It may also slow down or hinder the enzymatic breakdown of transmitters or affect the reuptake processes. These subsequent alterations in neural activity and behaviour provide an understanding of the functional significance of the targeted systems (Roth, 2016).

Applications of pharmacological studies:

Pharmacological methods are interestingly applicable across basic and clinical neuroscience. In animal models, they have been indispensable for working memory formation, why reward feels rewarding, and addiction. For example, one can inject a dopamine antagonist into the nucleus accumbens and observe the behavioural responses of an animal in a food search (Wise and Koob, 2014). In humans, it is difficult to just inject substances into the nucleus accumbens or other areas of the brain to watch what happens. Combining pharmacological challenges with imaging techniques such as PET or fMRI provides dynamic maps of neurotransmitter function, enabling the study of how serotonin signalling is quiet in depression or dopaminergic changes in Parkinson's disease (Smith *et al.,* 2017; Kringelbach *et al.,* 2020). An intriguing study by Webber *et al*. (2021) reviewed studies that gave drugs to healthy adults to explore dopamine's role in reward processing. The review justified the neurochemical basis of dopamine in motivation and reinforcement. Neuropharmacological applications, ranging from antipsychotics to antidepressants, also form the foundation of most contemporary psychiatric and neurological therapies.

Procedure in pharmacological studies:

Pharmacological studies involve several processes which include:

First pick the neurotransmitter target system of interest (dopamine, serotonin or glutamate) and also the receptor subtype that matters in the study. It could be the dopamine receptor subtypes 1 or 2, 5-hydroxytryptamine receptor 2A or 1A for serotonin, or N-methyl-D-aspartate receptor versus α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor in the glutamatergic system. The next step is to choose a pharmacological agent, which could be an agonist or antagonist, an orthosteric or allosteric modulator, or a reuptake inhibitor.

The second step is to determine the drug formulation, the route of administration and, more importantly, dosing. The oral route is best for human studies, while intraperitoneal injections are standard in rodents.

Thirdly, it is to administer the drug under controlled environmental conditions. It is important that saline and vehicle control are given as controls to know the drug-dependent effects.

Assessment of the neural and behavioural response comes next. This monitoring may involve the use of other electrophysiological or neuroimaging techniques (PET or fMRI), as well as behavioural testing (Morris and Macdonald *et al.,* 2015).

Data analysis is the last step, and it includes comparing drug vs. control conditions to determine the functional role of the targeted neurotransmitter system. Then evaluate dose–response relationships, onset/offset dynamics, and specificity of effects (De Rossi *et al.,* 2015).

Advantages and limitations of pharmacological studies:

The major advantage of pharmacological methods is their ability to provide causal evidence about the involvement of neurotransmitter systems in behaviour and cognition (Webber *et al.,* 2021). They are also relatively straightforward to apply and can be combined with electrophysiological or imaging techniques for multimodal insights (Gupta *et al.,* 2022). However, these methods have key limitations: systemic drug administration often lacks spatial specificity, leading to widespread rather than localised effects. Furthermore, drugs may have off-target actions, variable metabolism, and limited temporal resolution compared to techniques such as optogenetics (Roth, 2016). Ethical considerations also constrain the extent of pharmacological experimentation in humans.

Table 2**:** Comparative table of techniques for studying brain functions

Technique	Spatial Resolution	Temporal Resolution	Invasiveness	Cell-Type Specificity	Causal Inference	Translational Potential
EEG	Low (cm)	High (ms)	Non-invasive	Low	Low (correlational)	High (routine clinical use)
fMRI	High (mm)	Medium (seconds)	Non-invasive	Low	Low (correlational)	High (widely used in humans)
PET	Medium (mm-cm)	Low (minutes)	Minimally (radiotracer)	Medium (molecular/receptor)	Low (correlational)	High (clinical diagnostics)
In Vivo Electrophysiology	High (single-cell/µm)	High (ms)	Invasive	Medium (with targeting)	Medium (correlational, but direct)	Low (mostly animal)
Fiber Photometry	Medium (population, ~100-400 µm)	Medium (seconds)	Invasive	High (genetic sensors)	Low (observational)	Low (animal-focused)
Calcium Imaging	High (single-cell/µm)	Medium (ms-seconds)	Invasive	High (GECIs)	Low (observational)	Low (mostly animal; emerging human)
Optogenetics	High (cell/projection)	High (ms)	Invasive	High (genetic)	High (manipulation)	Medium (early human trials, e.g., vision)
Chemogenetics	High (cell-type)	Low (minutes-hours)	Invasive	High (DREADDs)	High (manipulation)	Medium (potential for systemic ligands)
Lesion Studies	Medium (region-specific)	N/A (permanent)	Invasive (or natural)	Low	High (necessity test)	Medium (observational in patients)
Pharmacological Interventions	Low-Medium (systemic/local)	Variable (minutes-hours)	Variable (invasive injection or systemic)	Medium (receptor-specific)	High (modulation)	High (drugs in clinical use)

(Adapted from Roth, 2016; Buzsáki *et al.,* 2012; Sandrone *et al.,* 2014; Khanna *et al*., 2015; Jun *et al.,* 2017; Garcia *et al.,* 2022; Rong *et al.,* 2023; Simpson *et al.,* 2024)

Discussion

The ten techniques examined here exemplify the range of approaches available for researching brain function. Their variations also highlight the importance of using approaches that are consistent with the research question. Because they are safe and simple to use, non-invasive methods like fMRI, PET, and EEG are crucial for human research. The EEG is excellent at capturing fast brain activity, but it has poor localisation, whereas the fMRI offers detailed spatial imaging but has low temporal resolution (Table 2). PET's capacity to provide molecular information is restricted by the use of radioactive tracers. These limitations highlight the benefits of combining techniques like EEG–fMRI that leverage both temporal and spatial strengths.

Invasive methods come with trade-offs, but they show things that non-invasive methods cannot reach. Electrophysiology is still the gold standard for temporal resolution; the electrodes catch every spike as it happens every millisecond. However, calcium imaging trades some of that precision for its ability to observe population dynamics for as long as weeks. Fibre photometry enables real-time tracking of activity in freely moving animals. This is where calcium imaging and electrophysiology intersect. Optogenetics and chemogenetics supplement these approaches in a different way by allowing the researcher to take control, although they come with limitations, such as genetic tweaks limiting their practicable use. Although these approaches are still in their infancy, they are very beneficial for comprehending mechanisms in animal models.

Lesion and pharmacological techniques continue to provide causal evidence, linking traditional and contemporary neuroscience. They are particularly useful for confirming novel techniques. However, careful interpretation is necessary, as off-target drug effects and brain compensation can confound them.

The significance of multimodal integration is a crucial finding in these comparisons. By combining recording and manipulation techniques or by employing both invasive and non-invasive methods, researchers can overcome individual limitations and gain a more complete picture of brain activity. Going forward, converting preclinical research into clinical practice will require developments in genetically tailored tools, minimally invasive devices, and higher-resolution imaging.

Artificial intelligence has started creeping into every aspect of neuroscience. It cleans up noisy data, picks out patterns humans would miss, and stitches together information from EEG, fMRI, and PET scans into something resembling a coherent picture. AI is tireless, unbored, and unbiased compared to humans. Xu *et al*. (2024) showed last year just how much better multimodal diagnostics are when machines help sort through the tedious process.

Hardware is catching up too. Wireless miniscopes mean animals no longer need to be tethered to recording equipment. They just move, explore and behave normally, and the data keeps coming in. Optoelectronic systems let you stimulate and record at the same time, closing the loop between watching and doing. PET-MRI systems at 7 Tesla are already here, giving structural, functional, and molecular images all at once (Cho *et al*., 2008; Judenhofer *et al*., 2008). Quantum sensors are on the horizon, and maybe one day they will let us see cellular activity without cutting into anything. Digital twins of whole brains are being sketched out, models that might one day let us simulate a tumour, a stroke or a drug before ever touching a patient (Xiong *et al*., 2023).

More recently, optogenetics and chemogenetics are inching toward humans. Lüscher *et al.* (2025) wrote about it. First-in-human trials for psychiatric conditions are at the clinical trial stage. All of these raise questions we are not quite ready to answer. Who owns your brain data? What happens when someone can read it without your permission? If we can enhance cognition, who will have access? The technology is moving faster than the ethics, and that should worry all of us. Such progress makes neuro-rights frameworks necessary and important.

Conclusion

A methodological toolkit comprising non-invasive imaging, invasive circuit-level probing, and causal interventions by lesions or medication is essential for studying brain function. Each method has its advantages: Electrophysiology and calcium imaging show cellular activity, optogenetics and chemogenetics allow direct manipulation, fMRI provides comprehensive spatial maps; and EEG enables real-time dynamics. Nevertheless, none of these methods is adequate on its own. Their true worth becomes apparent when viewed as complementary, creating a framework to tackle the intricacies of brain structure and function.

It is anticipated that neuroscience will progressively embrace integrative approaches that combine general non-invasive procedures with specific invasive techniques, including traditional methods such as lesions and pharmacology, into contemporary multimodal frameworks. This combination will improve the transition from research to therapeutic applications and deepen our understanding of fundamental neuronal functioning. Such an all-encompassing perspective underscores that neurophysiology is still evolving and has the potential to transform basic research and medicine.

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Authors' contribution

ENN, JAA, MI, ATA, AAA, SSI and MAG worked on the first draft of the manuscript. WIA and BVO provided the guidance and worked on the final version of the manuscript.

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