ERA-NET NEURON II

Symposium

~Synaptopathies~

Wednesday, May 14th, 2014
Bonn, Germany

Hotel Bristol
Prinz-Albert-Str. 2
53113 Bonn

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This Symposium is part of Work Package
Development of a strategic Research Agenda of the NEURON II project,
Work Package Leaders are Inserm and CNRS

Funded by the European Commission

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Welcome

Dr. Marlies Dorlöchter (PT-DLR, NEURON Chair, Bonn, Germany)

The symposium on "Synaptopathies" was launched with some welcoming words from Dr. Marlies Dorlöchter (PT-DLR, Bonn, Germany) on behalf of ERA-NET NEURON.

Introduction

Dr. Etienne Hirsch (INSERM, Paris, France) and

Dr. Bernard Poulain (CNRS, Paris, France)

Dr. Hirsch presented the symposium's general objectives, which had to do with reviewing the latest hot findings of research related to Synaptopathies, from basic to clinical neuroscience. The goals were to review and discuss the:

1) Normal functioning of the synapses
2) Major methods to study the function and dysfunction of the synapses
3) Mechanisms at the origin of synaptopathies in neurological, psychiatric and sensory organ disorders
4) Possible strategies to treat synaptopathies
5) Strategies to reinforce research on synaptopathies
6) Opportunity for a call for research on synaptopathies.

The areas to be covered by the international scientific experts in relation to the specific elements within the area of Synaptopathies were then introduced.

Understanding the normal and pathological synapse is a great challenge. The human brain is amazingly complex. The connectome is comprised of 100 billion neurons, wired with axons and dendrites, and connected with synapses. Yet, Dr. Hirsch made the point that we cannot understand the brain if we do not know how many synaptic types there are in the brain nor do we understand what a synapse is. And understanding the pathological brain will require understanding what a sick synapse is. For that we need the most advanced knowledge on the synapse. Synaptic protein composition shapes the functional properties of the synapse, the relationship between genetic variance and synaptic properties. What defines their robustness? Why does modifying a single protein, lipid, glycosylation process or single component lead to illness?

The ERANET-NEURON II Symposium on Synaptopathies provided a unique opportunity to brainstorm on the issue of understanding brain pathologies at the synaptic scale.

Normal Synaptic Function and Plasticity

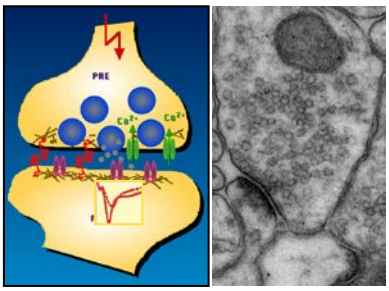
Dr. Eckart Gundelfinger, Leibniz Institute for Neurobiology (LIN), Magdeburg, Germany

Roughly 100 trillion connections make up the human brain's network. These synapses link about 100 billion neurons together with axonal wiring to transmit and integrate complex information. In addition to all these neurons, there is nearly an equal amount of glial cells. At the single synapse level, a complex architecture of molecular building blocks structures each junction to give it the necessary signaling elements that render it functional. Differences exist between the synapses at the protein level between brain regions and neuron cell-types. Disparities at the protein level also exist between normally functioning brains and those affected by neurological or mental health disorders. How individual synaptic connections in the brain are organized at the protein level is still an unsolved question in the field of synaptic neuroscience. Signaling molecules are continuously altered under normal brain function to enable rewiring and learning to take place. There is still much research to be done to determine how mutations in synaptic genes lead to brain disorders. The major themes dealt with in this talk involved the molecular organization at the pre- and post-synaptic level in both normal and diseased states, as well as the links between brain disorders and plasticity.

Synaptic junctions

Synaptic junctions are composed of four cellular components: the presynaptic bouton, postsynaptic apparatus, synaptic cleft, and perisynaptic astrocytes. Astrocytes and microglia play a role in the development of the synapse and also in regulating potential diseased states. Proper functioning at the synaptic level is necessary for information processing and learning at the circuit level. The underlying

proteins that make up the synapse are therefore central players. The synaptic junction is indeed filled with networks of proteins. The number has been estimated at 2000 proteins per synapse, based on proteomics.
Dr. Gundelfinger focused on the chemical synapse in the brain as a molecular machine for neuronal communication. At the chemical synapse, information arrives with an incoming action potential. This is a signal for calcium (Ca2+) influx, which signals the release of neurotransmitter from synaptic vesicles at the pre-synaptic level into the synaptic cleft. The neurotransmitters diffuse across the synaptic cleft and bind to receptors at the post-synaptic level, which activates the post-synaptic neuron. At the chemical synapse, electrical signals are converted to chemical signals, and then lead to an electrical response. Both pre- and post-synaptic components can be modulated to give an enhanced synaptic response, or the signal can be turned around, thus inhibiting the response. This kind of synaptic modulation, called synaptic plasticity, also affects the brain's 'integrative power,' as its many synapses concert together.



The chemical synapse.
Werner Zuschratter, LIN

Delicate balance in the brain

A delicate balance is needed in the brain for normal synaptic function. Too much excitation in the brain can lead to disorders like encephalic epilepsies, while too much inhibition can also lead to forms of mental impairment (e.g. Down's syndrome). Synapses can be classified as either excitatory or inhibitory. A vast majority, about 70-80%, of the synapses are excitatory, using glutamate as neurotransmitter; 20-30% are inhibitory and depend on GABA and glycine as neurotransmitters.

Disease genes and their proteins

Disease genes and their proteins are also being revealed. In the case of cell adhesion, molecules within the peri-synaptic extracellular matrix make the synapse's specificity. Asymmetry between the cell adhesion ligand and receptors, however, can in some cases trigger Autism Spectrum Disorders (ASD) (e.g. neurexin/neuroligin pair). At the post-synaptic terminal, mutations in the gene encoding the scaffolding proteins ProSAP/Shank have been linked to ASD and mental retardation. Mutations in the pre-synaptic Piccolo protein have been correlated with major depression and intellectual disabilities. Also in the presynapse, mutations in Bassoon have been linked to epilepsy and sensory (visual/hearing) impairments.

The Presynapse

a) Overview on the synaptic vesicle cycle at the active zone

Presynaptic release of neurotransmitters by synaptic vesicles occurs within a sequestered region of the pre-synaptic terminal, the active zone. A series of tightly-regulated molecular signaling events regulate the positioning and function of the synaptic vesicles. To give a brief overview of the synaptic vesicle cycle, postulated in 1995 by Dr. Thomas Südhof (now Nobel laureate), SVs containing neurotransmitters are first recruited to the synaptic membrane. There, vesicle docking and priming occur. Vesicle fusion to the synaptic membrane occurs. Upon the right Ca2+ signal, the vesicle releases neurotransmitter into the cleft. Then, vesicle recycling happens by mechanisms of exo- and endocytosis coupling or by direct retrieval of neurotransmitter. The events and molecules involved in this membrane trafficking cycle are the substrate for pre-synaptic plasticity events.

b) Composition and organization of the synaptic vesicle

A major recent breakthrough has been the identification of the quantitative proteome of the synaptic vesicles. As membrane-bound structures, they have a diameter of 35-50 nm and contain the classical neurotransmitters (in contrast to dopamine, serotonin or neuropeptides). They include all the proteins they need for the cycle to occur and the energy-demanding enzymes that pump H+ transporter proteins. The organization of the synaptic vesicle cycle has the been the subject of recent research in many labs. An elaborate three-dimensional structural model of a synaptic vesicle at the active zone has been rendered.

c) Recruitment and priming of synaptic vesicles.

How is the exact localization of specific subtypes of Ca2+ channels organized? The pre-synaptic molecule Bassoon has been found to be involved in this particular process. This mechanism regulates the distance between the Ca2+ channel and the release site, a determining factor of synaptic strength. The distance

between Ca2+ channels and the release site matters because once Ca2+ comes in, the concentration rapidly decays.

d) Exocytosis and endocytosis and activity-dependent recycling

Many proteins involved in the exo-endocytotic cycle (> 200) have been identified, and connections between them have also been uncovered. Exo-endocytotic coupling is based for the most part on the action of cytoskeletal matrix proteins. Exo- and endocytosis each occur in different compartments of the pre-synaptic bouton. While exocytosis happens at the membrane, endocytosis leads to reentry of the vesicles back into the matrix.

The Postsynapse

a) The excitatory post-synapse

As a model of the post-synaptic apparatus, Dr. Gundelfinger focused on the excitatory post-synapse. In the excitatory post-synaptic terminal, different types of glutamate receptors determine the performance of excitatory post-synapses. These are very important for plasticity. The most important receptor for strength is the AMPA receptor. For coincidence detection, there is the NMDA receptor. Both are ionotropic receptors, which means they are structured as channels in which (Sodium Na+) ions travel through when they are open. In contrast to the ionotropic receptors, there are also metabotropic receptors, such as metabotropic glutamate receptors (mGluRs), which modulate the whole system.

b) The post-synaptic density (PSD)

The PSD includes numerous signaling molecules, which play a functional role of adjusting the incoming signal, as well as a structural task in anchoring and localizing the ionotropic and metabotropic glutamate receptors. So, the PSD can also be considered as a substrate that responds to synaptic plasticity.

How is the PSD organized? The NMDA receptor core complex is fixed to the membrane and is localized exactly opposite the pre-synaptic release apparatus. Other receptors that can be found in the PSD include signaling molecules like ephrin receptor, insulin receptor—altogether contribute massively to the signaling. This complex molecular assembly at the PSD is important to keep in mind when looking at disease states.

c) Two layers of organization in the PSD

A first level of PSD organization is determined by scaffold molecules, which are closely coupled to the membrane receptors and ion channels. The second layer is comprised of ProSAP/Shank, ideally localized at the base of the PSD. Some of the molecules from this second layer interact with the actin cytoskeleton, to anchor the PSD.

The inhibitory synapse

At the pre-synaptic terminal, inhibitory synapses are similar to excitatory synapses, with differences mostly in the enzyme and vesicular transporter types. At the post-synaptic terminal, however, the inhibitory post-synpatic compartment has a distinct molecular architecture. Moreover, inhibitory synapses form mainly at the shaft of dendrites or on the neuronal cell body.

Subsynaptic networks

The post-synaptic regulatory network of excitatory neurons is very complex. Containing all the necessary proteins for post-synaptic signaling and modulation underlying synaptic function and plasticity, the signaling molecules make many connections, with communication between different signaling pathways. Dr. Gundelfinger formulated an analogy with the Paris metro map. Some proteins in this network are real signaling hubs and when something goes wrong it really affects synaptic function, but if something goes wrong in a less central region the effects are less drastic. In disease states, or under certain modified conditions, a part of the network might no longer function or be used. Altered signaling of a molecule can in some cases lead to a slight disruption in the signaling with most molecules functioning normally, or may have serious effects. ProSap/Shank proteins, for instance, are key in synaptic signaling. So if they are dysfunctional the synapse will likely be dysfunctional as well.

The perisynaptic extracellular matrix

The synapses in the brain are surrounded by a dense extracellular matrix (ECM). This net-like structure is found throughout the brain, especially around inhibitory neurons, and develops relatively late during

ontogeny. This peri-synaptic ECM plays an important role in regulating synaptic signaling and plasticity. By forming a net around the synapses, the ECM plays a role in stabilizing the structure of the synapse. This is important in the context of learning, since new connections are continuously being wired up and unneeded ones recede.
Receptors are mobile, can diffuse at the membrane by a process known as lateral diffusion and be exchanged between synapses. Single particle tracking techniques, which follow the movement of individual receptors at the neuronal membrane, are being used to study the effect of the ECM on receptor dynamics. Since receptor dynamics contribute to synaptic plasticity, which play a role in many disease states, being able to modulate their dynamics could be instrumental for clinical applications.
In a recent study from the Gundelfinger and Choquet labs, the receptor dynamics were monitored before and after removal of the ECM. Individual GluR1-containing AMPA receptors were followed using single particle tracking and the ECM was degraded by the activity of an enzyme. However, in regions along the post-synaptic density devoid of ECM, when the perisynaptic ECM was removed, there was an increase in the mobility of AMPA receptors and this altered short-term synaptic plasticity.
During the early stages of development, synapses are more open to forming new connections since the wiring is less stabilized and more dynamic than in adulthood. Certain brain disorders are linked to plasticity of the brain being either not flexible enough or on the contrary overly flexible.
Based on the ECM research findings, Dr. Gundelfinger's group came up with the hypothesis that the ECM may modulate the exchange between receptors inside or outside the synapse. They did experiments in cultured neurons, where they removed the ECM with the enzyme, hyaluase. In the control, the ECM was there. Then they removed the ECM and compared the dynamics of the very same receptor molecules before and after administration of the enzyme. The same receptor molecule explored a much larger region on the dendrite after removal of the ECM. This is also associated with changes in short-term synaptic plasticity, thus affecting synaptic function.
A very recent study from Dr. R. Frischnecht's group investigated the ECM's role in mediating plasticity during learning in the auditory cortex *in vivo*. The investigation's focus was on determining whether ECM degradation by an enzyme that acts on specific proteins could reopen the brain circuit's potential to learn a task that required high behavioral flexibility. In this case the task involved the tone discrimination of a specific frequency. Upon ECM removal, complex reversal learning was facilitated. This may be an event that happens in development. A lot of plasticity is needed for the connections to form initially. Subsequently they need to be stabilized, which is where the ECM comes in. Why put brakes on the brain's plasticity? One explanation is that if the brain remains flexible with constant restructuring of the synapses, it would work more slowly. So complex higher-order functions may come at the expense of neuronal plasticity. The first study to show that removal of the ECM could modulate plasticity in vivo in a sensory system was conducted by Pizzorusso et al 2002 Science. They showed that the rat visual cortex could be rewired after removal of the ECM.
Dr. Gundelfinger presented work done in collaboration with Dr. Choquet's lab, using single-particle tracking and fluorescence recovery after photobleaching (FRAP), to see by what mechanisms the ECM modulates stability vs. plasticity. They found that the ECM acted like a net, forming surface compartments on rat primary neurons, which reduced lateral diffusion of the AMPA-type glutamate receptors. Removal of perisynaptic ECM therefore increases the mobility of AMPA receptors.

The role of astrocytes at the tripartite synapse

Astrocytes are tightly associated with synaptic junctions, working alongside the pre- and post-synaptic terminals, in what is called a 'tripartite synapse'. Today it is accepted in the synaptic field that the astrocytes are essential players in synaptic transmission. This is reflected in both their structure and function. Functionally, the perisynaptic astrocyte removes neurotransmitters and K+ ions from the synaptic cleft and contribute to synaptic signaling and plasticity. The astrocyte includes the necessary molecules for neurotransmitter release. In addition, they produce some of the extracellular matrix components for the perisynaptic area and the synaptic cleft.
A single astrocyte can make connections with up to 100 000 synapses. Moreover, astrocytes can form electrical gap junctions between each other. Interestingly from a systems point of view, groups of

astrocytes can cover 1 mm^3 of synapses in the brain. What do they contribute to the circuit for information processing and how can modulating them help in treating patients with neurological disorders?

Dr. Gundelfinger pointed out the importance of astrocytes as dynamic cells within the nervous system, that change their function depending on the environment and whether the system is in a normal or disease state. The role and phenotype of the astrocytes influence synaptic plasticity. This is a matter of future research..
What's more, astrocytes have been found to produce extracellular matrix components. Dr. Gundelfinger described a mechanism for astrocytic implication in plasticity based on the idea when you remove the ECM, the available volume for transmission is greater. So the function of astrocytes in to context of synaptic function is still one that warrants attention.

The synaptic proteome during development and upon insult

The proteome changes during development, with the differentiation of neuronal types, forming different kinds of synapses, each assembling different sets of neurotransmitters. Synapses are not simply grouped into excitatory and inhibitory synapses. In fact, there is a high level of specificity. So far, no comprehensive study has considered the protein make-up of the broad diversity of synapses. Experimentally, if the synaptic machinery is highly degraded, new proteins are synthesized. It will be a crucial step to consider the changes in the synaptic proteome during learning and on the whole brain level, at different developmental stages and upon insult.

Synaptic plasticity

Synaptic plasticity can be seen as a shift in steady state equilibrium of synaptic protein turnover. Disease may affect this equilibrium and/or impair its maintenance. There are several modes of synaptic plasticity in the CNS, both structural and functional:

- Developmental plasticity refers to the plasticity mechanisms required during the making and wiring of the nervous system. One example has to do with the perisynaptic extracellular matrix (ECM) and its relationship with respect to the critical period of development. The critical period of the forebrain ends in humans at around 20 years of age. This coincides with the age of schizophrenia onset. So it is very likely that schizophrenia appears so late is due to a process within the wiring of the axons via the synapses.
- Classical Hebbian plasticity plays a role in coincidence detection and in processing short or long-lasting changes in synaptic transmission. Indeed, at the synaptic level neural activity can generate persistent forms of synaptic plasticity, like long-term potentiation and long-term depression of synapses (LTD or LTP). This long-term synaptic plasticity is the basis for associative learning principles. Short-term forms of plasticity play a role in facilitation, augmentation and depression (Paired-pulse) of synaptic function.
- Metaplasticity: Integrating the history of a synapse or an assembly of synapses, metaplasticity determines the predisposition to undergo dynamic changes.
- Pre- and postsynaptic modes: Correlated pre- and post-synaptic firing leads to LTP, allowing the presynaptic neuron to drive the postsynaptic neuron more strongly.
- Homeostatic plasticity is responsible for keeping the system in a physiologically modifiable range (e.g. synaptic scaling). This form of plasticity occurs at a more global level, within the neuronal network. When the circuit undergoes a general change in activity, it must adjust its general strength to the new activity state.

Conclusion

There is growing evidence of a convergence between several signaling pathways that are instrumental in maintaining synaptic integrity and protein homeostasis. Many recent publications from the labs of Dr. Gundelfinger, Dr. P Pedarzani, and many others, have linked proteome changes, the role of insulin-regulated pathways and synaptic plasticity. Importantly, it is now believed that aging leads to a reduction in synaptic protein homeostasis and widespread protein aggregation, whether or not related to disease.
Therefore, there is a need to tackle questions about normal neuronal function and plasticity using a systems-wide approach, while still paying attention to detail at the synaptic level. Details at the synaptic

level and with the whole-picture view of the neuronal network are required to understand the basis for brain function and neurological disease or psychiatric disorders. To do this, new collaborations are needed to bridge basic science research with clinical and applied research.

Innovative Methodologies to Study Synaptic Function

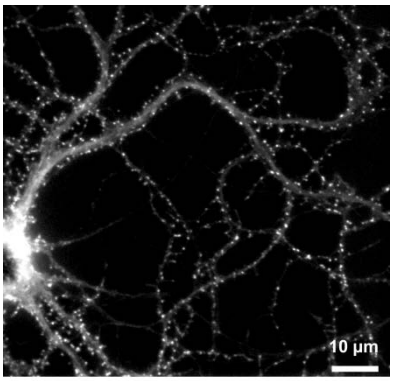
Dr. Daniel Choquet, CNRS, Bordeaux, France

Introduction

Each synapse is different from its neighbor. In a single neuron, each individual synapse has its own properties in terms of its protein constituents from the receptor apparatus to all kinds of molecular trafficking pathways. So there is a need to look at the synapses one by one. Accomplishing this depends on the development of new techniques that improve spatial and temporal resolution in order to detect and probe signaling dynamics at the single synapse and even the single-molecule level. Such techniques are needed to enhance the understanding of the networks and the mechanisms underlying brain plasticity, namely long-term potentiation (LTP) which strengthens the synapses.

The post-synaptic terminals along the neuronal dendrites, called the dendritic spines, are very complex, extremely small and dynamic. This makes dendritic spines extremely hard to study, especially at the single spine resolution. So these spatial and temporal considerations place huge constraints on the methods that can be used to study these neuronal processes. That said, once the tools are developed, they can be equally applied to study normal brain function or the underpinnings of synaptopathies, which are the diseases arising from or affecting synaptic dysfunction.

To look at something that is moving, like the dynamic spines, optical microscopy is the ideal technique. Optical microsopy, however, is limited by light diffraction. For example if pre- and post-synaptic components are stained in green and red, respectively, the synapses often appear yellow, due to light diffraction. New tools are needed in order to distinguish pre- vs. post-synaptic elements.



Post-synaptic dendritic sites (Homer-GFP)

Complex and dynamic organization of the synapse

On the one hand, the overall organization of synapses is very complex with many proteins interacting together so these synaptic signaling machines can operate. On the other hand, they are dynamic so the organization of synaptic networks moves all the time. Even in the basal state, the synaptic receptors undergo constant movement. The dynamic organization and function of the synapses must be addressed together. On the millisecond scale, exocytosis of synaptic vesicles occurs. Trafficking of receptors and movement of molecules inside spines occurs within seconds. And the plastic organization of the synapse moves all the time, adapting on the hour-long scale.

If we zoom to a single synapse, using super-resolution microscopy, we find that receptors are organized in small nanoclusters. The receptors move very rapidly between clusters, in both excitatory and inhibitory synapses. This has allowed us to draw up a very fine nanoscale model of the organization of the proteins in the synapse. The receptors in that synapse are not at all stable. They move within fractions of seconds in and out of the synapses. This has allowed to derive some general concepts on how receptors can be stabilized and changed from one dynamic state to another.

The dynamics of synaptic molecules are highly regulated by activity during the processes of plasticity, whether by short-/long-term plasticity or homeostatic scaling. Receptor movement can change, become faster or slower, regulated by activity and signaling molecules. In his talk, Dr. Choquet highlighted the

methods that can be used to study and understand these processes, as they related to normal neuronal function and disease.

• *Short-term dynamics:* In short-term plasticity, movement of these receptors participates in scaling the amplitude of the synaptic transmission and of the neuronal responses. When calcium concentration goes up, AMPAR and GlyR become more stationary and confined to the post-synaptic density. GABA receptor becomes more mobile and less confined. Receptor diffusion is at the basis of short-term synaptic plasticity, responding to fast synaptic transmission. The receptors are activated in a confined region, the post-synaptic density, just opposite the pre-synaptic the active zone release sites. So there is a constant receptor turnover at the post-synaptic density between the receptors that have already been activated and have entered a desensitized state and those new incoming naive ones. This rapid turnover allows for fast recovery after synaptic activation. If the desensitized receptors remain at the post-synaptic density, short-term depression persists.

• *Long-term dynamics:* The calcium-dependent phosphorylation or dephosphorylation events affecting accessory proteins and kinases signaling cascades with CaMKII, CAN, PKC and PTK is important in regulating the receptor dynamics for long-term plasticity. Long-term plasticity changes like long-term potentiation (LTP) or long-term depression (LTD) happen as a result of changes in the movement of the synaptic components. Modified signaling events, phosphorylation of the intracellular domains of receptors by different enzymes change the way the receptors traffick and cluster at the post-synaptic sites. In the case of NMDA-mediated LTP at excitatory synapses, these molecular changes lead to an increase in AMPA receptor content at the post-synaptic density. The turnover is therefore changed and accordingly the signaling molecules modify their activity to regulate the increase in glutamatergic AMPA receptors at the PSD. This increases the efficacy of the synapse. Conversely, during long-term depression, there is a loss of AMPA receptors at the post-synaptic density. The receptors diffuse out of the synapse, get internalized in the spine to eventually get recycled or degraded. The result is a weakening of the synapse. The synaptic scaffold elements can rearrange within minutes or hours, allowing changes in the efficacy of synaptic transmission.

Finally all the other elements of the tripartite synapse, the neighboring modulatory neurotransmitters and glial factors, secreted by peri-neuronal glial cells, regulate all these processes.

Approaches to study synaptic function & dynamics

• **Imaging**: A great variety of imaging techniques exist, providing numerous approaches to study synaptic function at different levels and scales. **Optical microscopy** is particularly well-suited for live synaptic imaging. **Electron microsopy** and **mass-spectroscopy imaging**—this is very new—certainly offer a lot especially in the context of **correlative microscopy**, in terms of understanding the whole synaptic structure down the the individual molecular complex organization.

• **Physiology:** Electrophysiology is of course a key technique to study neuronal function, through **patch-clamp recordings**, **micro arrays**, **optophysiology**.

• **Proteomics** and **Phospho-proteomics** are useful to determine the components expressed in given subtypes. **High resolution mass-spectroscopy** is crucial to determine differences in protein expression in different brain regions. This will be very important in order to decipher the specifics of small brain regions using a small number of animals to determine the various levels of expression of different proteins and how they are modified.

•**Genomics** are important for understanding disease. Being able to sequence large cohorts of individuals with **next generation sequencing** will be crucial.

•**Metabolomics** are now becoming more popular in the field of neuroscience since the energy consumption of neurons is extremely important.

•**Behavior:** Everything that happens at the synapse has a consequence in terms of behavior of the individual. It is therefore paramount when studying the effect of synaptic modulation to assess the behavioral effects.

•**High content screening**: This will certainly be important in order to understand how thousands and thousands of different molecules act together to regulate the synaptic transmission. If the goal is to ultimately treat disease, finding ways to screen for molecules with various roles at the synapse is very important.

•**Modeling synapse function:** The synapse is very complex. There are many molecules. To really grasp its complexity, creating models of how the synapses work is important to understand synapse function. Synaptic modeling can be done by **neurocomputation**, or by building smaller, simpler biomimetic systems – heterologous culture systems, substrate patterning, microfluidics to make artificial synapses.

Techniques to modify synaptic function at various levels

•**Modifying genes** with **spatio-temporally controled gene expression**, from **knock-in** mouse models to **single cell electroporation** or **single cell gene modification**. For instance, CRISPRs (*clustered regularly interspaced short palindromic repeats*) are DNA loci containing short repetitions of base sequences. siRNA and shRNA are extremely useful tools and may also be employed as therapeutic strategies.

•**Modify protein stability** is also a new avenue that is emerging. A few recent papers have been published describing the ability to disrupt targeted proteins. Looking more at the level protein networks there is now a large variety of ways to **disrupt protein interactions**, especially using **neuropeptides**. A number of peptides are now in phase 2 or phase 3 of clinical trials, e.g. for stroke. **Biomimetics** are also now being used to modify protein-protein interactions at the synapse.

•**Optical control** of synaptic and neuronal activity as a whole is now also being performed, with the growing panel of **optogenetics tools** and **ligand uncaging** approaches.

The revolution of Optogenetics: the light-gated genetically-encoded channelrhodopsin2/halorhodopsin have been used in a variety of neural systems to study the effects of activating/inhibiting neurons within complex networks. They can be applied *in vitro*, in slice physiology, and *in vivo.* Optogenetics tools can also be combined with other tools like two-photon glutamate uncaging. For instance, the Oertner lab (Basel, Switzerland) repeatedly paired glutamate uncaging pulses in single synapses identifed on spines by two-photon imaging with photostimulation of a channelrhodopsin 2. This induced LTP and swelling of the spines was observed.

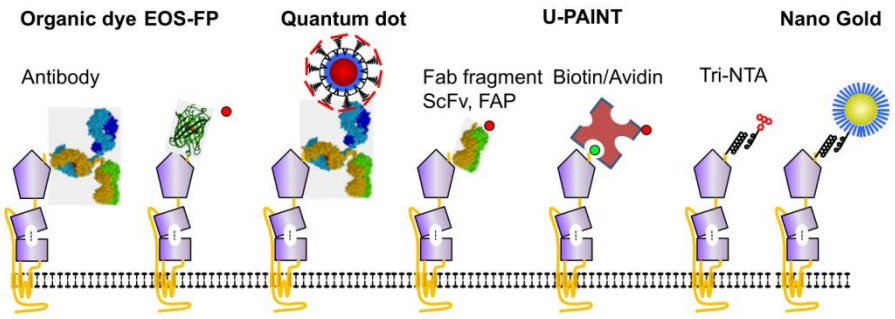
•**Modifying individual molecules**, such as receptors, channels and enzyme function, with the action of classical or non-classical pharmacology.

New labeling technologies

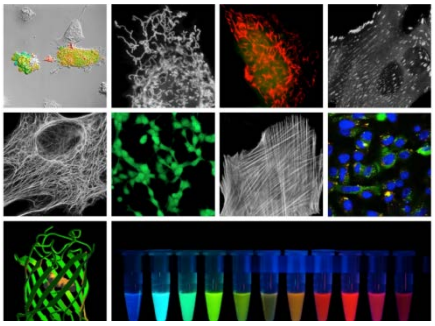
Observing and measuring the dynamic organization of synaptic components requires a way to label proteins in order to follow them. Old techniques like antibodies, protein-tagging with fluorescent proteins or organic dyes have existed for a long time. Given the diffraction limit in light microscopy, the size of the probes matters.

What are the **new labeling technologies**? At the level of **probes**, there are new **ligands** that can be used (e.g. antibodies, ScFvs, intrabodies, nanobodies, small molecule fluorescent ligands). Then, individual molecules and the probes themselves can be highlighted in order to follow them, by engineering fluorescent proteins, organic dyes or nanoparticles. It is also possible to modify the protein in order to visualize them with methods such as epitope tagging, GFP, **ligases** and **un-natural aminoacids**.

Techniques to label proteins for bulk or single molecule studies



New methods to label proteins for bulk or single molecule studies make use of photoactivatable or photoconvertible fluorescent proteins, e.g. *EosFP*, which undergoes photoconversion in response to illumination with a certain wavelength.



Quantum dots, fluorescent nanocrystals of semiconductor material, can be used for single particle tracking, by conjugating them to streptavidin or antibodies. Study from the labs of Dr. Choquet, Dr. Triller and others, individual receptors are labeled using single quantum dots through a targeted antibody. This probing system could be used to study receptor lateral diffusion in and out of synapses.

Photoactivatable fluorescent proteins

A variety of small probes (e.g. Biotin/Avidin, NanoGold and Tri-NTA) can be developed against specific synaptic targets and then linked to fluorescent proteins or dyes to reveal location or activity.

Directed evolution to generate new high affinity ligands

Fragment antigen-binding (**Fab**) fragments. Either using single chain antibodies derived from normal antibodies: single-chain variable fragment (*scFv*), FAP

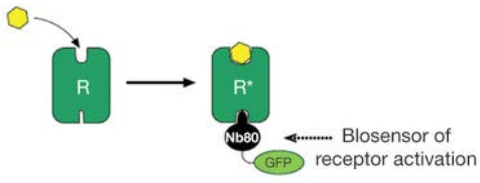
A whole generation of technologies has emerged for directed evolution to generate high-affinity ligands. Directed evolution depends on random mutant library generation and screening techniques to engineer or optimize functions of proteins. One class of proteins for which this process is particularly effective is antibodies, where properties such as antigen specificity and affinity can be selected to yield molecules with improved efficacy as molecular labels or in potential therapeutics. Recent progress has been made in generating intracellular functional antibody fragments, such as the camelid heavy-chain antibody, to target and trace cellular components in living cells.

Nanobodies

How can nanobodies be used to address questions about the synaptic proteins and their interactions with each other? In one recent study from the lab of Dr. M. Fukata, a single chain method was used to distinguish PSD-95 specifically when fused with a palmitoylation tag, a natural cellular process that tags proteins to the membrane. The authors developed a small nanobody in order to track the PSD-95 when palmitoylated and discovered that this tag keeps the PSD-95 proteins localized within clusters in the postsynapse. This in turn is important for anchoring AMPA receptors and for synaptic transmission. This research determined an important recycling event for post-synaptic organization and turnover, whereby local palmitoylation cycles define activity-regulated postsynaptic subdomains.

Conformation-dependent nanobodies

Another example of a recent research publication concerns the development of conformation-dependent nanobodies to detect activated receptors. Indeed the Von Zastrow laboratory and others directly probed for the activated form of the β2-adrenoceptor, a GPCR. This piece of work provided the first evidence that receptors keep on signaling even once it is internalized within the endosomes. This suggests the possibility of several types of signaling processes, depending on whether the receptor is at the cell surface or within the cytoplasm.

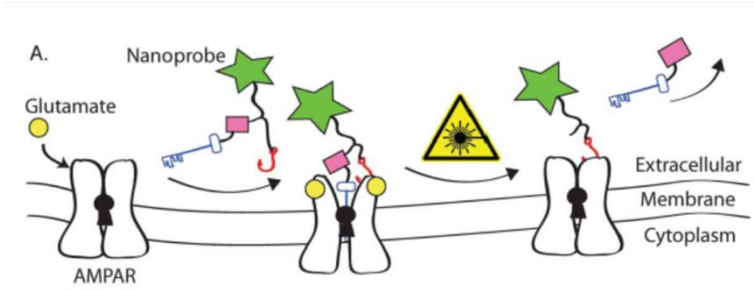


Biosensor of receptor activation, Irannejad et al 2013 Nature

Nanoprobes: Silent, fluorescent labeling of native neuronal receptors

Many of these probes depend on epitope-tagging, which can be made very specific, but they also come with artifact since the natural protein is not being looked at directly. What we are lacking for the future are endogenous probes. There is something very important to do in terms of labeling proteins endogenously with fluorescent proteins. One promising study, which came out of the group of Dr. JJ Chambers, introduced a minimally-perturbing ligand against an endogenous receptor. They were able to label and

visualize endogenous dendritic receptors on live neurons. They designed a ligand-directed probe targeted towards AMPA receptors that forms a covalent bond with the ion channel. The probe is designed so that photolysis of a portion of the probe leads to release of probe ligand from the receptor while still leaving the fluorescent tag on the receptor. In this way, a stable covalent bond is formed between the nanoprobe and the target receptor, producing the silent, fluorescent labeling of native neuronal receptors. This is to highlight an important frontier, which lies between biologists and chemists to develop tools to study synaptic function.



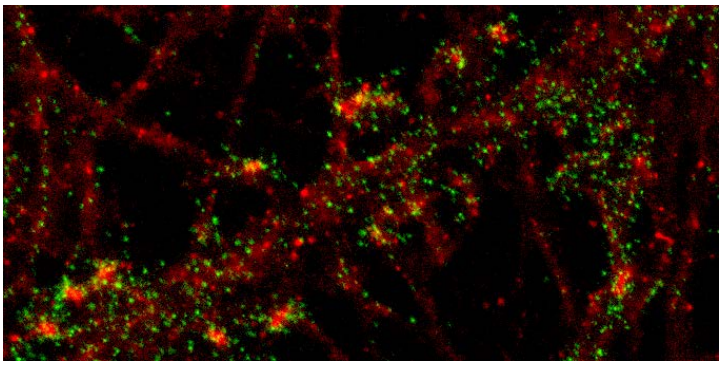
Silent, fluorescent labeling of native neuronal receptors
Vytla et al 2011 Org Biomol Chem

Microscopy techniques to study synaptic dynamics and organization

In terms of **microscopy**, there are many approaches to study synaptic function, starting with **crystallography**, or **fixed high resolution cryo EM** to really look at the protein structure. With the advent of **nanoscopy,** it is now necessary to increase the microscope's resolution.

L**ive microscopy** with the recent blossoming of numerous **super resolution approaches**, including **STED**, **PALM**, **STORM**, **SIM** and **uPAINT**, which are adapted to the size of synapses

a) STED (Stimulated Emission Depletion) Microscopy decreases the size of the fluorescence spot by deactivating the fluorophores surrounding the point of interest.
b) PALM (PhotoActivated Localization Microscopy) localizes single molecules based on the use of optical highlighter fluorescent proteins that stochastically switch on and off. By taking sequences of images over time, a superresolution image is formed. Single particle tracking of AMPA receptors was recently performed in Dr. Choquet's lab. Indeed they employed PALM to image neurons transfected with the GluA1 AMPAR subunit genetically fused with the photo-switchable fluorescent protein, mEos2, which switches from green to red emission. This gave a single-molecule readout that enabled the researchers to track the dynamics of each receptor. These state-of-the-art methods led to the observation that the AMPA receptors were confined to nanodomains.
c) STORM (Stochastic optical reconstruction microscopy) is another superresolution microscopy approach, enabling single molecule detection. Using two-color STORM, it is possible, for instance, to observe, at nanometer and millisecond resolution, partial colocalization of the glutamate receptor subunit GluA1 and post-synaptic protein PSD95, which stabilize the glutamate receptor nanodomain.



d) SIM (Structured illumination microscopy) is based over-sampling.
e) uPAINT (Universal Point Accumulation Imaging in the Nanoscale Topography)

The single molecule super-resolution uPAINT method records many single molecules at the surface of a cell by constantly labeling while imaging. uPAINT can be used with any wide-field fluorescence microscopy with oblique illumination to track ultra-high density single molecules on live cells.

Synapses must studied in vivo as well. We currently lack techniques to study individual synapses in vivo. So this is a good path for future technical development.
To investigate protein interactions, functional imaging approaches also exist, like **Fluorescence correlation spectroscopy (FCS)** and **Förster resonance energy transfer (FRET)**.
Looking at synaptic function, you actually have to look at the chemical signal transduction that occurs, preferably at the single-synapse level, if possible in live cells. In order to study molecular signal transduction at the single-spine level, you need reporters and so it is important to develop a variety of biosensors. The goal is to report either protein-protein interactions or signaling activity that you can use to image down to the single spine level.

- **FRET-FLIM** (Förster Resonance Energy Transfer-Fluorescence-lifetime imaging microscopy) is a well-established fluorescence-based system to study molecular interactions. Potential binding partners are each labeled with a spectrally distinct fluorescent probe. If binding occurs, the interacting proteins are at a distance of a few nanometers apart so that upon light stimulation at the wavelength specific to the donor, the donor can transfer its energy to the acceptor. In turn, the acceptor emits fluorescence and the donor's fluorescence lifetime decreases. The FRET-FLIM system can be used to look at scaffold protein interactions.

Monitoring Stargazin Interaction with PSD-95 based on FRET-FLIM

Dr. Choquet's group used a FRET sensor to report the interaction between a Stargazin (an AMPA receptor accessory subunit) and the scaffold protein PSD-95. This allows measuring the interaction directly in the individual synapse in live cells. The fluorescence acceptor, the fluorescent protein mCherry, was introduced on Stargazin (STG::mCherry). The donor, eGFP was inserted on the PSD-95 (PSD-95::eGFP). The fluorescent proteins did not affect the function of Stargazin or PSD-95.

An actin polymerisation state bio-sensor

The group of Dr. Ryohei Yasuda investigated actin polymerization in live cells by using a FRET pair consisting of mEGFP-actin monomer and the F-actin monomer labeled with sREACh (non-radiative YFP variant). Based on FLIM decay curves, they were able to determine the fraction of polymerized F-actin relative to the total monomeric actin in the cell. Interestingly, the fact that they found more binding in the dendritic spines with respect to the dendritic shaft indicated elevated levels of actin polymerization in the post-synaptic compartments.
Furthemore, the development of new biosensors is important to go toward all-optical techniques, with biological sensors able to report the metabolic or signaling activity of individual molecules (e.g. CaMKII, cAMP, ras, Calcium ...).
Optical strategies for sensing neuronal voltage, voltage sensors, are also being developed for opto physiology. Microbial light-driven rhodopsin charge pumps (e.g., halorhodopsin, archaerhodopsin) have been engineered to change the membrane voltage. Although this tool, pioneered by Dr. A. Cohen and others, holds great potential for minimally invasive functional studies of neurons, it is only available for *in vitro* cultured neurons. More work is needed before it can be used *in vivo*.

Conclusion

The next steps in synaptic research need to focus on unraveling the synaptic protein complex composition and organization at the nanoscale. This will depend on correlative microscopy and high resolution proteomics. In addition to this it will be elemental to study synaptic function as a joint effort between biologists, chemists, physicists and computer scientists, bridging the gap between molecular and integrated levels. New methods will also need to be developed for all optical measurements of synapse activity and enzymatic functions, such as new bio- and voltage sensors. Optical tools to control protein traffic and organization, in vitro and in vivo, are also needed. Improved gene delivery and manipulation methods will also be essential. Finally, to study of the role of specific molecules in the assembly of synapses biomimetic systems can be designed, which can be tightly controled, down to the single synapse connection, and assayed with live optical imaging to study the synaptic dynamics.

Age-Related Synaptic Alteration

Dr. Pierrette Gaudreau ; Université de Montréal, Québec, Canada

Introduction

In humans, successful aging is defined by low probability of disease, high cognitive and physical function and active engagement in society. Yet aging often engenders altered emotional behaviors, such as increased anxiety. Research indicates that exposure to stress and obesity accelerates cellular aging. This may lead to cognitive dysfunction and increased risk of dementia. A central challenge in the field that studies aging is to determine the molecular and cellular processes that lead to successful aging. This challenge is not easy to tackle due to the complexity of the nervous system. Many factors, both intrinsic (genetic) and extrinsic (e.g. food intake, stress), influence the way we grow old, age itself being a factor. A strategy to address the issue is to focus on successful aging within the context of a specific environmental or genetic alteration, and by making use of appropriate animal models. Recent research based on rodent models has focused on the behavioral (i.e. memory, anxiety) and neurochemical (i.e. glutamatergic signaling, prodynorphin expression) effects of aging.

In this talk, Dr. Gaudreau proposed a conceptual framework in which Group 1 metabotropic Glutamate receptors, Homer proteins, related signaling pathways and immediate early gene expression play a crucial role in learning and memory formation in the aging brain. She based her presentation on studies of three different animal models that are relevant for elucidating the relationships between neurochemical and behavioral processes within the context of aging.

1) Successful aging based on inter-individual differences in the Long-Evans rat strain

The effects of aging vary from one individual to another. Basing themselves on exactly these inter-individual differences, researchers have been able to distinguish two groups of aging animals in one rat line (called Long-Evans): aging-impaired vs. aging unimpaired rats. The researchers introduce a memory test to the rats (Morris Water Maze) with fixed criteria (e.g. learning curve, reverse memory, latency to task completion) to determine whether they are successfully or unsuccessfully aging.

Synaptic plasticity and successful aging

Synaptic plasticity is important for maintaining cognitive performances associated with successful aging. Indeed the nervous system has evolved ways to reinforce the strength of certain synapses (long-term potentiation, or LTP) and silences others (long-term depression, or LTD). These mechanisms depend, at least in part, on the density of AMPA receptors that are recruited to the post-synaptic terminal at the synapse. Behavioral performance in aging animals correlates with Glutamate NMDA receptor-dependent LTD in young animals and Glutamate NMDA receptor-independent LTD in aged animals. This implies that cognitive abilities may be sustained in aged individuals by a switch in mechanisms of synaptic plasticity.

Glutamate receptors and successful aging

Group 1 metabotropic Glutamate receptor (mGluR) plays a key role in successful cognitive aging. The comparison of memory-impaired versus -unimpaired aging Long-Evans male rats revealed differences in the density of glutamate receptors at the post-synaptic density (PSD). PSD levels of mGluR1a and mGluR5 are reduced in aging-impaired animals, in comparison with the aging-unimpaired group. Furthermore, dysfunction of mGluR could be a major player in neurodegenerative disorders like Alzheimer's disease. In Alzheimer's disease, ß-amyloid aggregates, one of the characteristics of Alzheimer's disease, block mGluR-dependent LTD leading to decreased plasticity-dependent cognitive functions.

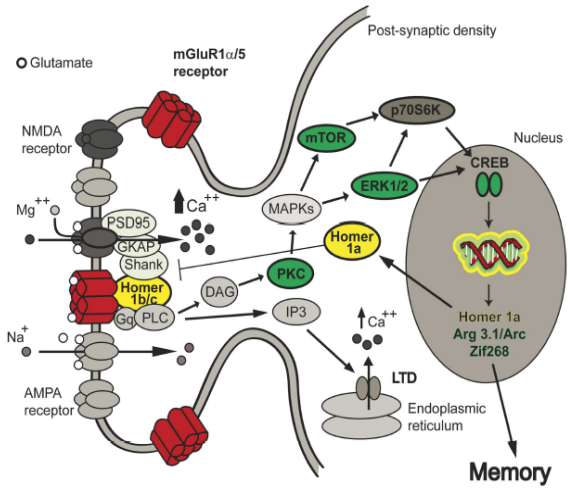
Downstream signaling of mGluR1 and mGluR5: Interaction with scaffold protein Homer

The downstream signaling pathway of mGluR1 and mGluR5 depends in part on its association with Homer scaffolding proteins. Homer interactions indeed regulate mGluR-dependent plasticity. Moreover, the ratio between Homer isoforms 1a:1b/c is closely tied to mGluR-mediated plasticity Homer 1a acts by uncoupling mGluR5 from the translational machinery.

mGluR5 and Homer1 colocalize in the hippocampus in both memory-impaired and memory-unimpaired aging rats. Both mGluR5 and Homer 1 were expressed and co-localized less in the hippocampal dendrites of aging-impaired compared to aging-unimpaired rats. Homer 1a-c colocalized less to the post-synaptic density in memory-impaired rats. Additionally, the ratio Homer 1a:Homer 1b/c was significantly higher in the post-synaptic densities and synaptosomes of aging-impaired rats, with respect to aging-unimpaired rats. In the hippocampus of aging unimpaired rats, mGluR binds Homer1b/c (the longer Homer isoforms), maintaining activation of crucial signaling pathways.

Immediate early gene expression

Downstream Homer, the mGluR signaling pathway controls the expression of immediate early genes that are expressed in response to recent neuronal activity, i.e. after spatial memory training. In terms of the signaling pathways, numerous enzymes are associated with mGluR activation. More specifically, the PKC and mTOR phosphorylation levels correlate with good memory performances in aging rats based on the Morris Water Maze test.



mGluR and Homer proposed signaling pathway Ménard & Quirion 2012 Frontiers in Pharmacology

In the hippocampus of aging-impaired rats, Arc expression is reduced and significantly correlates with Morris Water Maze performance: this is in line with lower mGluR-mediated signaling. Sustained expression levels of the immediate early gene, Arc/Arg3.1, and colocalization with the neuronal marker, map2, during aging also correlated with memory-unimpairment. Studies such as this one are aimed to map out the molecular interactions within the synapse, so as to understand behavior.

2) Stress in aging leading to cognitive dysfunction

Dynorphins and aging

Dynorphin (Dyn) is an endogenous opioid peptide, which plays a role in controlling emotions and stress-related behaviors. During aging, increased levels in dynorphins have been associated with aging impairment. For instance, increased dynorphin levels in the hippocampus have been associated with impaired spatial memory. Given the negative effect of increased dynorphin levels, researchers investigated whether downregulating dynorphin improves cognitive abilities in an aging mouse model. To do this, Dr. Ménard and colleagues used a knockout mouse that carried a deletion of the prodynorphin gene, the precursor of dynorphin, the Pdyn KO mouse. Pdyn KO mice exhibited unimpaired navigation, spatial memory and reverse learning. These results made the Pdyn KO mouse a good model to study the underlying mechanisms of memory function.

Downregulation of dynorphin gene expression protects aged mice from cognitive impairment

Given the link between increased dynorphin levels in the hippocampus and effects on memory and navigation, spatial memory was assessed in the Pdyn KO mice. Their performance in the Morris Water Maze was taken as a measure of their spatial memory. Comparison of Pdyn KO and wild-type mice at various stages in aging revealed memory impairment in older wild-type mice compared to young mice and both young and old Pdyn KOs. Furthermore, non-spatial memory can be assessed based on recognition of novel objects. Old wild-type mice spend the same amount of time with new and familiar objects, in contrast

with old Pdyn KO mice which behave similarly to young mice. This means that upregulation of dynorphin prevents the ability to recognize new objects.
Low anxiety is a measure of unimpaired cognitive function and successful aging. Anxiety can be tested experimentally in a behavioral task, such as the elevated plus maze or open field test, in which mice are evaluated on how freely they explore elevated spaces or open spaces to reach food (considering their natural aversion to open spaces). In the elevated plus maze, old PdynKO mice, explore the open spaces more than old wild-type mice (no difference is noted in young animals). Aged-induced anxiety behaviors are thus reduced in Pdyn KO mice. Hence, increased Pdyn levels during aging enhance age-related anxiety.

Upregulation of group 1 mGluR in the aged brain and expression of plasticity-related genes

Recent studies have discovered links between deficient Group 1 metabotropic glutamate receptor (mGluR)-mediated LTD and cognitive dysfunction affecting memory. Comparing mGluR levels in old Pdyn KO and old wild-type mice, reveals enhanced mGluR, specifically mGluR1α and mGluR5, in the hippocampus and cortex. Intact mGluR function can be assessed by applying a mGluR-sepcific agonist, which triggers mGluR-mediated LTD, a silencing form of plasticity in Pdyn KO mice. Interestingly, this does not happen in old wild-type mice or young mice.
Moreover, formation and consolidation of memories involve the expression of immediate early genes such as Homer1a and Arc. The levels of protein encoded by these immediate early genes decrease gradually with aging in the wild-type mice hippocampal formation but are maintained in old Pdyn KO. Immediate early gene expression levels further correlate with cognitive function, as assessed in behavioral tasks such as the Morris Water Maze. Based on the Pdyn KO successful aging model, the plasticity-related genes Homer 1a and Arc appear to be correlated with cognition in aging.
Evaluating the effects on cognitive function of pharmacological modulation of the group 1 mGluR pathway could lead to potential therapeutic strategies. Pharmacological treatment with either the mGlu5 receptor agonist, CDPPB, or norbinaltorphimine, an antagonist for the dynorphin-targeted κ-opioid receptor, reverses the cognitive deficits found in old wild-type mice, making them memory-unimpaired. In contrast, the mGluR5 antagonist, MPEP, which prevents mGluR-mediated signaling, leads old Pdyn KO mice to become memory-impaired (Menard et al., J. Neuroscience, 2013). Whether the relationship between Homer1a and Homer1bc is altered in this model is still an open question.

3) Obesity in aging

Interindividual differences and sexual dymorphisms related to aging

An increased incidence in recent decades of obesity and diabetes in older adults is a major public health concern. Resistance to obesity and low incidence of chronic disease are marks of successful aging. Understanding the pathways that may trigger resistance is important to understand successful aging.
Dr. Gaudreau presented an aging model, the obesity-resistant LOU rat strain (Louvain/C/Jall), that displays intact spatial, recognition memory, enhanced longevity, low anxiety, decreased stress-related Pdyn expression and intact synaptic plasticity. The LOU rat strain lives over 1.5 times longer than common rat strains. LOU rat body weight are lean (at 12% body mass) despite *ad libitum* feeding, in contrast to the Sprague-Dawley common rat strain which becomes obese at 20 months. What is the cognitive and anxiety status in aging LOU rats? Is it possible to emulate the healthy aging characteristics in Sprague Dawley rats by decreasing their body weight in a calorie-restricted model? These are central questions addressed by the Gaudreau group.
Sexual dimorphism must be taken into account when studying the impact of cognitive impairment in neurological disease. Among the LOU rats, there is a sexual dimorphism with regards to longevity with a median lifespan at 34 months for males and 38 months for females. Moreover, old males display less intact memory than females, in the reverse probe.

Obesity resistance and unimpaired cognitive function

In terms of spatial memory, old *ad libitum*-fed LOU rats perform similarly well as young ones based on learning platform position in the Morris Water Maze. Moreover, reversal learning and retention functions are intact. In comparison, old obese *ad libitum*-fed Sprague Dawley rats exhibit impaired learning acquisition. These effects can be prevented by calorie-restriction.

Recognition memory is intact in the LOU rats, as they perform well in novel object recognition, spending more time around novel objects than familiar ones. In the case of old Sprague-Dawley rats that were not dieting, however, recognition memory deficits could be noted. Dieting therefore prevented cognitive deficits.
Dr. Gaudreau's group has examined the effect of long-term calorie restriction on recognition memory, which causes the Sprague-Dawley rat to respond similarly as the obesity-resistant LOU rats. Moreover, calorie restriction also minimizes the effects of aging on increased prodynorphin levels. This is demonstrated by the fact that *ad libitum*-fed obese rats express increased levels of Pdyn in the hippocampus and this is not the case in the old obese-resistant LOU strain, or in young rats in general.

Glutamate receptor signaling is associated with successful aging in obesity resistant rats

Age-related weight gain affects expression of glutamate receptors and immediate early genes in response to neuronal activity. The hippocampal CA3 region is very important in encoding pattern recognition and there is a tight relationship between glutamate receptor function in this region and the inter-individual differences in cognitive function during aging. Dynorphins in this area could affect glutamate transmission and therefore lead to impaired synaptic plasticity underlying memory processes in the old *ad libitum*-fed Sprague Dawley rat brain. Group 1 metabotropic glutamate receptor 5 and immediate early genes *Homer 1a* and *Arc* expression were unaltered in aging LOU rats but lower in obese old *ad libitum*-fed Sprague Dawley than old calorie restricted rats.
Indeed, Group 1 metabotropic glutamate receptor 5 (mGluR5) expression is associated with successful cognitive aging in rodents. As Dr. Gaudreau showed previously, mGluR5 levels were increased in aged Pdyn KO mice. Pdyn levels were unchanged in LOU rats up to 42 months. In contrast, mGluR5 levels decreased significantly in old memory-impaired obese Sprague-Dawley rats but this reduction could be prevented by long-term caloric restriction.
Furthermore, the mGluR5-related signaling is reduced in the obese old adult. Nutritional intervention such as adult-onset long-term caloric restriction could prevent age-induced reduction of glutamate receptor expression and signaling in Sprague-Dawley rats.
However, this does not allow the complete preservation of youthful spatial memory. Can diets inducing obesity (high-fat/high-glucose diet) in common rat strains also affect the LOU rats' predisposition for successful aging (cognition and/or lifespan)? How does this work in the hippocampus of the LOU rats?
In addition to this, age-exacerbated anxiety and induced memory deficits in Sprague-Dawley rats, anxiety was high in old obese Srague-Dawley rats, as measured by performance in the Elevated Plus Maze. The test, taking place in a plus-shaped apparatus, elevated above the ground with two arms that are open and two that are closed, is based on the animals' aversion to open areas, and tending to remain in the closed arms rather than in the open ones. Obese old *ad libitum*-fed Srague-Dawley rats were very anxious and never entered the EPM open arms. In contrast, long-term caloric restriction reduced stress-related behaviors and increased locomotor activity. In the other model of successful aging, the LOU rats exhibited lower anxiety and traveled over greater distances on the open arms. Conversely, the LOU rat anxiety level was not affected by aging up to 42 months of age. Total locomotor activity however increased.
In the open field test of anxiety very old LOU rat distance travelled in the open field center area was lower than in other groups, and locomotor activity was intact. In contrast, old obese *ad libitum* fed Sprague-Dawley rats were very anxious and never entered the open field's central area. In a similar way to the LOU model, long-term caloric restriction reduced stress-related behaviors.

Conclusions

Rodent models indicate that successful and healthy aging is associated with intact memory function and low anxiety. Healthy aging depends at least in part on intact glutamatergic signaling (via mGluR5) and low Pdyn expression into advanced age. Reduced expression of the prodynorphin gene protects from age-related cognitive decline and anxious behaviors. In fact, it does this by maintaining group 1 mGluR expression, function and related signaling, which in turn mediates synaptic plasticity needed for memory and other cognitive functions.
Dr. Gaudreau pointed out the need throughout the talk to discuss data in quantitative terms noting that principal component analysis and correlation matrices can also be used to compare many different factors

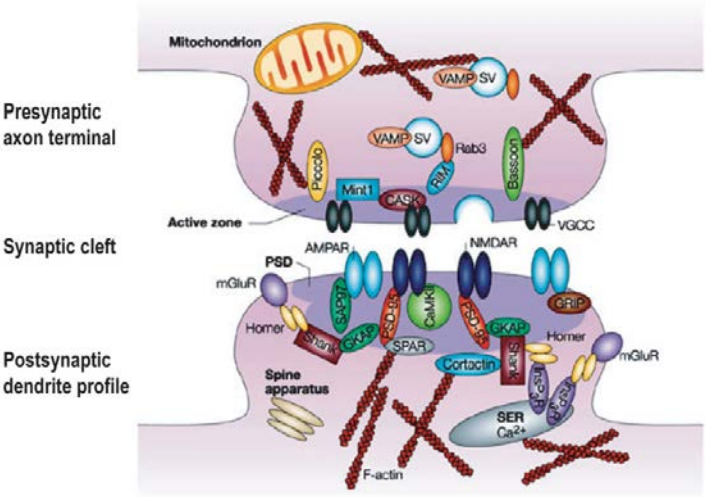
at once. For a future call, Dr. Gaudreau put forth the need to consider sexual dymorphism in animal models and human subject research. In addition to supporting a diversity of animal models, Dr. Gaudreau highlighted the need for diverse technologies to address the secrets to successful aging, which she called "a gigantic puzzle."

Synaptopathies in Neurological Disorders

Dr. Monica Di Luca, University of Milan, Milan, Italy

Defects at the synaptic level are seen in a very large number and variety of neurological disorders, from common to rare diseases, and developmental to aging disorders. On a grand scale, the cost of brain diseases in Europe is close to 800 billion euros. This represents an enormous burden on society. In these sorts of neurological diseases (migraine, epilepsy, dementia, stroke, Parkinson's disease, Multiple Sclerosis), a vast majority of cases (almost 90%) has a synaptic dysfunction. From a semantic point of view, the term 'synaptopathies' was introduced to neuroscience in the last few years to designate some key pathogenic features of a large number of brain diseases. Dr. Di Luca defined synaptopathies as human disorders caused by defects in synapse formation or function. Dr. Monica Di Luca discussed synaptopathies as they relate in particular to neurological disorders.

The synapse is very complex under both normal and abnormal functioning. Turning to the excitatory synapse as a model synapse, we see that this is a highly organized structure as a consequence of a protracted developmental program, whereby the synapse is first assembled, organized and stabilized in the mature individual. In the mature synapse, however, some flexibility and plasticity is maintained. Dr. Di Luca referred to the synapse as an 'individual organelle,' a macrodomain of the neuron with intrinsic properties, which are the following: capacity to exert local membrane and protein trafficking, local metabolic activity, local modulatory signaling cascades fostered by the large network of scaffold proteins with protein-protein interactions and local protein synthesis. So already at the individual synaptic level, there is a high degree of complexity.



The excitatory synapse as a model synapse (Chiu and Cline Neural Development 2010)

While studying the single synapse is important, a broad research focus is needed account for the diversity between the different synapses, since it also reflects the complexity of nervous function. We need to scale up our concept of how a synapse works because it doesn't work on its own but instead in a network. The network activity governs neuronal function and our behavior. So we need to merge the concepts of synaptic function with our perception of what happens at the circuit level.

The vision of the synapse is further complicated by the long-lasting changes that occur. At the level of the dendritic spines, permanent alterations are encoded by gene transcription. These long-lasting changes depend on a signaling pathway from the spine to the nucleus to control gene transcription. Special messenger molecules, originally characterized by Dr. M Kreutz, travel from the synapse to the nucleus. In the nucleus, they regulate transcription factor activity and gene expression. How these spine-to-nucleus signaling aspects are affected in disease is an open question. This complexity, at the synaptic or network level, is prone to being disrupted in the diseased state.

What are the causes of synaptopathies? Altered number and shape (dysmorphogenesis) of the dendritic spines (post-synaptic compartments) can give rise to decreased cognitive function and dysfunction of the synapse. A large number of mutations in the genes that are related to the structural and functional organization of the synapse, i.e. the synaptome, have been associated with synaptopathies. Mutations are

not necessary. Even in absence of a mutation, synaptic dysfunction may occur with problems in the pre- and/or post-synaptic architecture. Perturbing just one protein can lead to general problems in synaptic function. Synaptopathies can arise from early dysfunction of normal synaptic processes, i.e. local trafficking, membrane receptors' availability, alterations of local metabolic capability and molecular complexity. Are these changes the cause or the consequence of the disease? We still need research to address this major question.

Spinal dysmorphogenesis as a cause of synaptopathies

Synaptopathies can arise from a dysmorphogenesis of the spine. The number of dendritic spines reflects the number of excitatory synapses and provides a good measure of variability between different neurological pathologies and with respect to normal development. Under normal development and aging, the dendritic spine number increases from birth throughout childhood and beginning at adolescence stabilizes, proceeding to slowly decreasing during the course of the individual's life. In developmental diseases, such as autism spectrum disorders (ASD), enhanced numbers of spines are present, even before the symptoms emerge. Focusing on Alzheimer's Disease, a typical aging disorder, a sharp decrease in the number of spines occurs, resulting from a lack of spine maintenance in late adulthood. This lower number of spines says something about a deficiency in the connectivity. An important question posed by Dr. John Fiala and Dr. Kristen Harris is whether altered dendritic spines are the intrinsic cause of the accompanying neurological disturbances or a consequence (or 'compensatory response') of the disease. A lot of connectivity research is needed to determine this.

Genetics as a cause of synaptopathies

Synaptopathies can arise from mutations in the synaptome. A pivotal study from the group of Dr. Seth Grant demonstrated that if one looks at the synaptome of the post-synaptic density alone, mutations in the genes encoding these proteins are related to over 130 primary brain diseases. These mutations have been found to arise from over 200 post-synaptic density genes.

A number of synaptic genes/proteins mutated in neurological and psychiatric disorders are already known and have been well-characterized by the labs of Dr. Bourgeron, Dr. S. Grant and Dr. C. Bagni, among others. Mutations in the gene encoding the synaptic scaffolding protein, SHANK3, for example, are associated with ASDs. So this is an example of a mutation in a single gene, which plays such an important synaptic role that at the network level, the brain does not function properly. Moreover, a new function for the fragile X mental retardation protein (FMRP) has recently been described in regulating PSD-95 mRNA stability. This provides a mechanistic underpinning for the effects the mutation of FMRP has in fragile X syndrome, an ASD. Finally, a last example Dr. Di Luca gave regarding single gene mutations, which lead to neurological disorders, was the neurologin gene. In another study from the labs of Dr. H. Ehrenreich and Dr. N. Brose, neuroligin-3-deficient mice were characterized as a model of a monogenic heritable form of ASD with an olfactory deficit. These various examples of research studies show that neurological disorders can arise from the synaptic assembly being perturbed by a single genetic mutation.

Synaptopathies can arise from early alterations in the function and dynamics of the proteins in the microdomains of the pre- and post-synaptic compartments. These may occur during the early prodromal phases of disease. They may also occur in sporadic forms of neurological disorders in which there is no neurodegeneration. Perturbing the molecular complexity can affect neurotransmitter release. For instance in the case of glutamate receptors, mGluRs, these dynamic proteins can travel in and out of the post-synaptic density and even between spines. The many processes involved in the cycling (activation, internalization and recycling) of a single receptor protein show the complexity of the synapse and explain how synaptopathies can arise in many different ways.

Dr. Di Luca gave some concrete examples of neurological diseases concentrating two major disorders of aging: Alzheimer's disease and Parkinson's disease. These multi-modal pathologies start out with dysfunction at the synaptic level before evolving into diseases characterized by cell death and neurodegeneration. She stressed that these are two examples of pathologies that still require research in the area of synaptopathies. Alzheimer's and Parkinson Disease represent a grand challenge for the next decade. Both diseases can be examined as synaptopathies based on spine dysmorphogenesis and alterations in the complex synaptic assemblies.

Azheimer's disease is characterized by dysfunction of the synapses

A decrease in dendritic spines in the early asymptomatic phase eventually leads to cognitive dysfunction in Alzheimer's disease. Dr. Di Luca referred to an experiment in which her research group exposed hippocampal neurons to newly formed ß-amyloid aggregates taken from human Alzheimer's patients. This led to dendritic spine abnormalities.

Amyloid-ß aggregates are a key characteristic of the disease. In very early stages they take the form of soluble oligomers. Interestingly, the number of spines decreases significantly even when exposed to soluble oligomers of ß-amyloid in vitro. Dr. Di Luca stressed the fact that many other studies had also described this (from her own group, that of Dr. Sabatini, Dr. Mandelkow, Dr. Mulle, and others). There is a real need for translational research in which information from preclinical studies is then verified in the patients, from bench to bedside. Indeed the decreased number of spines that has been studied in Alzheimer's disease patients has been found to correlate with the degree of cognitive impairment, depending also on the stage of the disease.

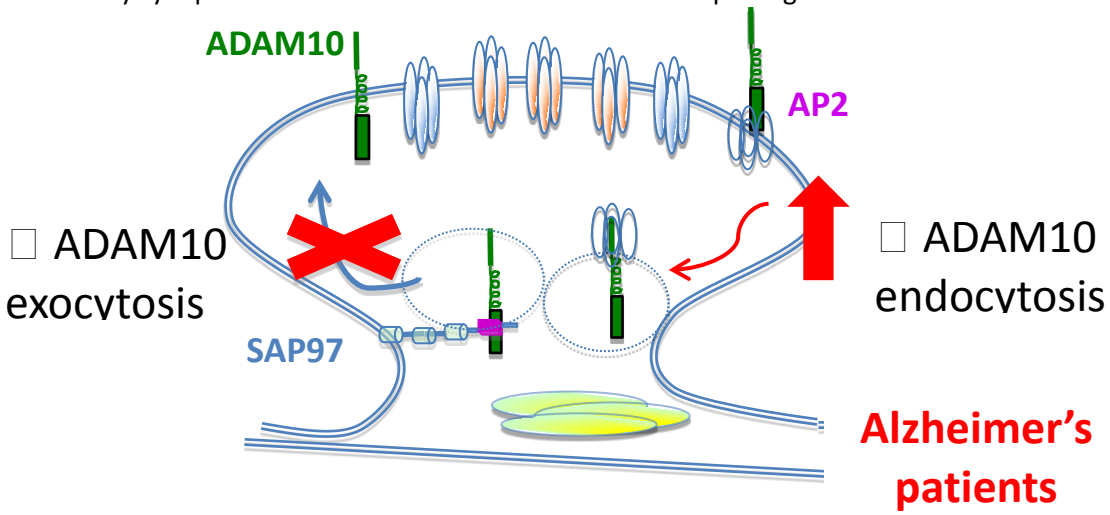
As a consequence of the decreased spine number in AD, plasticity and flexibility of the synapses within the circuits are also affected. This also has a molecular counterpart. Many different research groups have contributed to this work investigating the effect of ß-amyloid aggregates on synaptic plasticity. As described in a recent review by Dr. Lynn Raymond (University of British Columbia) and colleagues, ß-amyloid can also affect astroglial release of glutamate. Strategies based on promoting astroglial glutamate release could have an impact on the extrasynaptic receptor that has a signaling cascade that is different from the synaptic glutamate receptor. It is also possible to impact the synaptic receptor and change the availability of the number of active receptors to the membrane. This is true for NMDAR, for instance, which can be internalized or subtracted from the spine. Again, it is also possible to impact synapse-to-nucleus signaling pathways. For instance, it is known that, upon activation of NMDA receptors, the post-synaptic density component Jacob is sent to the neuronal nucleus. The nuclear signaling events eventually shape synaptic function and control NMDA-receptor–induced cellular degeneration. Alzheimer's disease has also been associated with Jacob dysfunction. There is this idea that in the early stages of the disease the ß-amyloid aggregates are impacting different molecular aspects of the synapse.

Involvement of intrinsic factor, ADAM-10, in Alzheimer's disease (AD)

Many studies indicate that synaptic dysfunction in Alzheimer's disease is not only the consequence of the exposure of the synapse to amyloid-ß, but is also an intrisic property of the diseased synapse. A major research goal is to find ways to modulate the dynamics and local trafficking of intrinsic factors inside the synapse, so as to regulate the ß-amyloid aggregates.

In a study from Dr. Di Luca's lab, an enzyme that produces ß-amyloid was found to be an intrinsic element of the spine and of the synapse. The enzyme they studied, A disintegrin and metalloproteinase 10 (ADAM-10), has the property of preventing the formation of ß-amyloid and is enriched in the post synaptic density of excitatory synapses. A natural process removes ADAM10 from the membrane by clathrin-dependent endocytosis. The researchers managed to identify an ADAM-10 partner that regulates the enzyme levels at the membrane where it is activated. This partner is in fact a clathrin-associated protein, called AP2. In the hippocampus of Alzheimer's patients, the association of ADAM10/AP2 occurs at higher levels than in healthy individuals. Furthermore, in hippocampal neuron cultures undergoing long-term potentiation, ADAM10 experienced AP2-mediated endocytosis. Conversely, long-term depression induced ADAM10 regulation at the synaptic membrane where the enzyme was active. Moreover this process depended on association with another partner, the synapse-associated protein-97 (SAP97), and prevented formation of the ß-amyloid. The data related to ADAM10 provide a mechanism controlling the enzyme's localization and

activity at excitatory synapses that is relevant to Alzheimer's disease pathogenesis.



ADAM10 trafficking/endocytosis mechanisms are impaired in AD patients

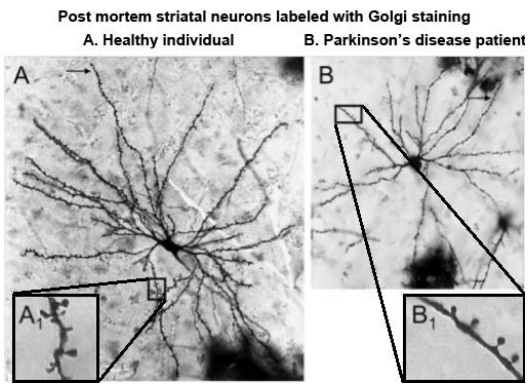
In fact, in this work, the researchers recapitulated the amyloid-ß cascade inside the synapse. They demonstrated that the ß-amyloid aggregation was not just an effect of factors extrinsic to the synpase, but instead that the synapse itself has an intrinsic capability of modulating the production of ß-amyloid. Proteins regulate the exo-endocytotic pathways that determine the dynamic of the availability of the enzyme at the membrane. For future research it is important to study the trafficking of many proteins and enzyme at the synapse.

The areas related to Alzheimer's disease that need research focus include those associated with spinal defects and ß-amyloid formation. In terms of early synaptic dysfunction and ß-amyloid aggregation, the molecular and cellular pathways involved still need to be revealed. It is difficult to know which comes first, whether the ß-amyloid aggregates or the synaptic dysfunction. This needs to be uncovered in order to find therapies. Moreover, research must focus on understanding local trafficking, which regulates both synaptic function and ß-amyloid formation.

Dr. Di Luca made the point that research must be scaled up from the perspective of the individual synapse to the systems-level. Future research projects should be aimed at figuring out ways of remapping the circuit, as well as fostering plasticity and a proper excitation/inhibition balance.

Synaptic, cellular and molecular underpinnings of Parkinson's Disease

Switching the focus to Parkinson's disease, Dr. Di Luca made parallels with Alzheimer's in terms of spine dysmorphogenesis. Synapses are very diverse depending on where they are located. In the case of the striatal medium spiny neurons, there is a significant loss of spines in Parkinson's patients. On the head of these neurons' dendritic spines, excitatory cortical cells form synapses, and on the neck of the spines dopaminergic neurons in the substantia nigra, affected by the disease, make contacts. So in Parkinson's disease, compared to Alzheimer's disease, there is greater complexity due to the convergence of the two synapses on a given spine. For instance, a change in dopamine levels can alter the post-synaptic composition of glutamate receptors.



Decrease dendritic branch and spine number in Parkinson's disease (Stephens et al 2005 Neuroscience)

Modification of NMDA receptor composition may characterize Parkinsons' disease

Altered dynamics and composition of the ionotropic NMDA glutamate receptor in this particular striatal circuit is a synaptic trait of the disease. Modified assembly of NMDA receptors has been found across species, in 6-OHDA-lesioned rats, in MPTP monkeys (Collab. M. Di Luca & J. Obeso), but also in Parkinson's patients (Collab. M. Di Luca & E. Hirsch).
A particular research conducted by Dr. Di Luca's group focused on the effects of dopaminergic denervation in the striatum. They used a rat model of Parkinson's disease, the 6-OHDA-induced partial-lesioned rats, characterized by loss of dopamine and partial denervation of striatal neurons. Whole-cell electrophysiological recordings in brain slices revealed loss of NMDA-dependent long-term potentiation (or LTP) but normal long-term depression (or LTD). NMDA receptor composition was assessed by protein purification. A dramatic increase in the NR2A subunit, with overall NMDA R levels unchanged, suggested that a reorganization of the post-synaptic density was occurring. This altered synaptic plasticity and motor behavior. However they proceeded to "rebalance" the homeostatic organization of the synapse and composition of NMDA receptor subunits using a cell-permeable TAT peptide fused to the NMDA receptor NR2A subunit (TAT2A). Plasticity and motor behavior could effectively be restored. They now need to study this in other species and bring this to clinical trials.

Major issues at stake

Dendritic spine defects are shared in Alzheimer's and Parkinson's disease. An open-ended question has to do with whether the spinal dysmorphogenesis is a by-product or the driving force of the pathology. The neuronal circuits and molecular pathways that are directly and indirectly involved, need to be identified. In Alzheimer's, early synaptic dysfunction and formation of amyloid-ß aggregates appear to come hand in hand. This is an area worth further investigating. Similarly in Parkinson's identifying which cell-type is at the origin of the altered synaptic plasticity will be crucial to understand disease onset. Furthermore, it seems that the point of convergence between two pathways stands as the pillar of pathogenesis, holding potential as potential new therapeutic targets. Developmentally, more work is still needed to understand the early events in synaptic dysfunction. It is crucial for the next step to study synaptic function and dysfunction at different scales, in order to view the whole synaptic protein network and thus identify convergent/divergent pathways between different synaptopathies. New tools then become necessary to allow for an integrated view of crossed pathways in major neurological diseases.

Synaptopathies in Psychiatric Disorders

Dr. Claudia Bagni, Vlaams Instituut voor Biotechnologie, Leuven, Belgium

Cellular and Molecular Aspects of Mental Disabilities and Psychiatric Disorders: Insights from the Synapses

Intellectual disabilities follow from a large group of neurodevelopmental, neuropsychiatric disorders, as well as neurodegenerative disorders. Typical neurodevelopmental disorders include Fragile X syndrome, Autism Spectrum Disorders (ASDs), schizophrenia and Major Depressive Disorder (MDD), ADD/ADHD, etc. Intellectual disabilities are normally associated with deficits in two or more areas of adaptive behavior (e.g. communication, daily living skills, social skills, self-care, health, security, leisure). Intellectual disability is defined based on an IQ below 70. The IQ test was first used in the early twentieth century, and although it is often called into question, it still prevails in the clinic. Onset of intellectual disability usually occurs before the age of 18.

Prevalence of neurodevelopmental and psychiatric disorders

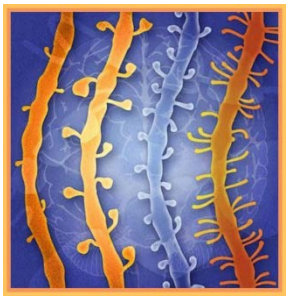
Worldwide, 10-20% of children and adolescents experience mental disorders (2013 WHO Report). Half of all mental illnesses begin by the age of 14 and three-quarters by the mid-20s. There is a developmental phase during childhood and adolescence from ages 8-10 that is critical in determining how mental disorders present clinically. Neuropsychiatric conditions represent the leading cause of disability in young people all over the world. If left untreated, these conditions can severely perturb the child's development, limiting their educational achievements and potential to live full and productive lives. The incidence of schizophrenia is 5-11/1000; that of Major Depressive Disorder (MDD), 4.3% and bipolar disorder, 1/100,000. Some neurodevelopmental and psychiatric disorders, like Autism Spectrum Disorders, vary greatly in their prevalence from one country to another (highest in North Korea and the USA at 1/38 and 1/68, respectively, compared to 6.2/1000 worldwide).

What's at the bottom of the disorders?

The brain is the organ of behavior and also the organ of our minds. What happens at the level of our neurons, that are locus of our memories, determines our behavior. The frontal lobes are the brain's largest structures and have been associated with a large number of disorders. These include Attention Deficit Hyperactivity Disorder, schizophrenia, and bipolar disorder, which are disorders of the prefrontal cortex.There are many connections between brain areas whose dysfunction entails behavioral disorder.
Within the different brain regions, the working units are the neurons and glial cells. The neurons extend processes called neurites (the dendrites and axons) that enable communication from one brain region to another. On a micro-scale, the dendritic surface is lined with small spines that make up the post-synaptic components needed to receive and process information from the pre-synaptic terminals of partnering cells. Unhealthy spines therefore can lead to serious intellectual disabilities.

Dendritic spine morphology in development and mental health disorders

Over time, the morphology of dendritic spines matures during development from a more elongated shape to one that looks more like a mushroom. Their structure varies anatomically from one brain region to another, and also changes depending on physiological conditions. With intellectual disabilities, the spine's maturation is arrested in the elongated shape. Incomplete spine maturation is called 'dysmorphogenesis.' This information is not new. Already in 1897, Lord Sherrington pointed out that "...each synapsis offers an opportunity for a change in the character of nervous impulses." This concept, later defined as plasticity, underlies our basic assumption about learning, memory, early childhood education and even stroke recovery. There are many disabilities associated with spinal dysmorphogenesis. Some are due to genetic mutations. Environmental factors can also affect the spines. If we challenge our brains later in life, dendritic spine morphology and function can change, for better and for worse. With malnutrition, alcohol abuse, epilepsy, sensory deprivation, those spines will become dysmorphic, regress, and become long and thin, taking on an immature shape. In contrast, an experientially enriched environment can mature dendritic spine morphology and improve function.



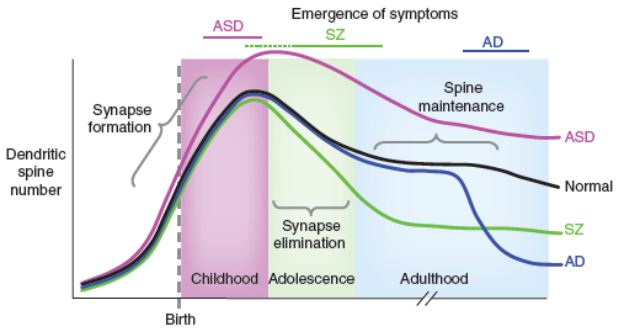
During spine development, the pre-synaptic terminals become larger and acquire the machinery necessary for neurotransmitter release, which can be studied electrophysiologically and morphologically. As spines mature, three main events take place: receptor trafficking, actin remodeling and local protein synthesis.

Image Credit: Uta Mackensen, EMBO
Changing shape of dendritic spines in development and dysmorphogenesis

Local protein synthesis shapes and reshapes the synapse

Long, highly polarized cells like neurons have evolved a sophisticated way of regulating gene expression. On the one hand, protein can be synthesized in the cell body and then transported to distal locations like the synapse. On the other hand, this strategy to control the synaptic proteome entails that long distances must be traveled. This is inefficient considering the changes that must occur quickly in synapses in short-term learning as well as long-term memory. The dendrites contain all the components needed to translate and

synthesize new proteins; this theory was first put forth by Dr. O. Steward (Irvine) and colleagues in the 1980s.
mRNA exits the nucleus, assembles with specific RNA-binding protein, travels through axons and dendrites in a translationally-silent manner, reaches the synaptic compartment and then only after synaptic stimulation are those mRNAs loaded on the polyribosomes (clusters of ribosomes) and translated into proteins. This is a fast way to respond to a stimulus through potentiation of the spines. If the neurons were to rely on the first strategy, they would have to first send the signal back to the nucleus, transcription, translation, protein transport all the way down. This would take much longer than having the RNA already at the dendritic spine, waiting to be translated. It is also a way of differentiating one synapse from another. So we have here a mini-compartment without a nucleus that can respond differently in space (brain regions) and in time (development).
The work of many years shows that this mechanism exists and plays a major role during neuronal development. Genetic alterations of the molecular pathways implicated in the control of local protein synthesis contribute to Intellectual Disability (ID) and Autism Spectrum Disorder (ASD) syndromes.



Importantly, spines experience a life-cycle during development and maturation of the individual, increasing in number from early development to adolescence, and then slowly decreasing throughout adulthood. Dendritic spine deficiencies occur in certain disabilities, with both enhanced and lower numbers, depending on the disorder. The synaptic view of the disorder is that the spinal number and dysmorphogenesis are major pathogenic factors.

Penzes et al., Nat. Neuroscience 2011
Dendritic spine number and emergence of symptoms in healthy and affected individuals

A lot is already known about synaptic protein composition of nearly 2000 proteins. An estimated 150 of these have been associated with neurological diseases. What is now needed is continued research to build an understanding of the structure-function relationships of proteins and protein interactions during development. This is key to figure out what goes wrong in the associated synaptopathies.
One of the prototypic neurodevelopmental disorders is Autism Spectrum Disorder (ASD). ASD is a very complex disease. Patients can be more mildly or severely affected, hence the notion of 'spectrum.' Some of the characteristic features include an inability to interpret other people's emotions and social isolation. The core symptoms are impaired social interactions, deficient communication and repetitive behaviors. ASD patients therefore function on what is believed to be an altered cognitive level. The prevalence of ASD is high, estimated worldwide at one child in 160 (WHO estimate).

Genetics and the environment: causes of Intellectual Disabilities

ASD, and many other intellectual disabilities, are multi-factorial disorders cause by environmental and genetic factors. Most neuroscience research has focused on the genetic factors, although environmental factors also play a key role. Environmental factors affecting ASD in particular include *in utero* viral infection, *in utero* alcohol exposure (from alcohol abuse by the mother), Bisphenol A and exposure to Mercury.
Over the last three years, a number of studies have described candidate genes associated with neurodevelopmental disabilities (e.g. ASD and schizophrenia) based on exome sequencing (i.e. sequencing the part of the genome made of the protein-coding genes or exons). 203 genes have been identified with *de novo* mutations from individuals with ASD: a relatively high percentage (14%) of the genes is associated with a synaptic function and moreover, these are interconnected. This reinforces the idea that synaptic proteins do not each function individually but instead as a network. They depend on protein-protein interactions.

The many interactions between proteins imply that many different sub-synaptic components are actually converging in their molecular signaling cascades. This protein network entails communication between vesicles, adhesion proteins, molecules responsible for signal propagation and those that stabilize the synapse.

Fragile X Syndrome: case of dysmorphogenesis

The discovery that Fragile X Syndrome (FXS) is a monogenic disease is currently changing the way researchers and clinicians view ASD. FXS is the most common form of inherited intellectual disability. It is due to the mutation of an X chromosome gene, fragile X mental retardation 1 (FMR1) that codes for the RNA-binding protein FMRP. Until ages 3-4 when synaptogenesis is still going on, children with FXS do not display any feature abnormalities. There are slight behavioral signs but no major early symptoms. When FMX patients become adults, they display more evident signs, e.g. elongated face and low IQ. Other cognitive and behavioral symptoms consist of aggression, self-injurious and obsessive-compulsive behavior. The clinical and imaging signs, including macro-orchidism and seizures, overlap with other synaptic diseases. All these marks of the disease make FXS very complex. Despite FXS being a monogenetic cause of ASD, the disease manifests itself on a broad spectrum. Not all FXS patients have the same symptoms. Some high functioning FXS patients are able to work, while other FXS patients are severely affected. Of course genetics are the leading cause of the disease but the environment also participates in determining how the disease will unfold.

Patients with FXS have elongated immature spines due to a genetic mutation. Interestingly, this kind of dendritic spine dysgenesis can also be found in other intellectual disabilities with no diagnosis. The spinal dysmorphogenesis can be seen at any age. Mouse and fly models have been developed to study the FXS disease. This has allowed to move forward and even to enter clinical trials.

Why are the spines dysmorphic?

CYFIP1's dual role regulating local protein translation and actin remodeling

Dr. Bagni presented work from her lab that shows that the RNA-binding protein (FMRP), together with its partner cytoplasmic FMRP-interacting protein 1 (CYFIP1), puts a brake on the local synthesis of a subset of synaptic proteins. These proteins are involved in controlling processes like actin remodeling and receptor internalization. If the brake is not there (i.e. FMRP or CYFIP1), more proteins are synthesized. This contributes to the elongated spinal morphology characteristic of FXS. CYFIP1's importance as a key synaptic element has become very clear within the field of intellectual disability. Dr. Bagni's group and others have worked to better understand the mechanism by which CYFIP1 contributes to proper synaptic function.

The gene encoding CYFIP is located on chromosome 15q, not far from a region implicated in other ASD-related syndromes. This region is highly unstable and, of the FXS patients that have been studied, CYFIP seems to be at the crux of the disability. Microduplication or microdeletion containing CYFIP plus another three genes leads to cognitive disabilities, schizophrenia, epilepsy, autism. CYFIP1 interacts with RAC1 small Rho GTPase, is involved in actin polymerization and lamellopodia formation and regulates protein translation in mammals. Through many years of research, it has become clear that CYFIP1 plays a dual role in two apparently unrelated processes, inhibiting local protein synthesis and favoring actin remodeling.

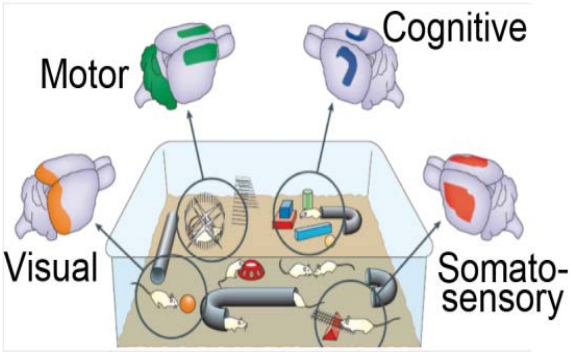
Dr. Bagni's group identified two CYFIP1-dependent complexes, one involved in protein synthesis, the other in actin remodeling. Both are necessary for spine formation and maintenance. The single particle tracking techniques (in collaboration with Dr. Choquet's lab) determined that the molecule could change confirmations. Dr. Bagni proposed that if researchers could dissect the CYFIP1 interactome, all the proteins with which CYFIP1 interacts, this would increase the knowledge about FXS and all the other related intellectual disabilities. 38% of the genes within the CYFIP1 interactome are also associated with schizophrenia; 22% with autism. This makes CYFIP1 a linking molecule between the different intellectual disabilities.

Bridging the knowledge between basic research and clinical applications

Basic research investigating synaptic function and dysregulation in fly, mouse and other organisms can be extremely informative and bring insight into the mechanisms underlying disease. There is also a need to bridge the knowledge acquired from basic research with patient-based research. For instance, Dr. Bagni's

group worked extensively to identify Fragile X syndrome patients with specific CYFIP point mutations. They identified a patient with one allele of the gene completely deleted and the other having a specific point mutation. From there, they were able to generate patient cell lines with the mutation. That is when they were able to confirm the link between the Fragile X Syndrome and the defects in actin polymerization, cell morphology and protein synthesis. So from neurons that are studied using mouse models, knowledge can be applied to screen for patients. Then, with patient-derived somatic cell experimentalists can confirm that the same molecular pathways are affected.

Now, 23 years after FXS was first discovered in 1991, much progress has been made in understanding the root causes. Searching for therapeutic intervention targets, mRNA-related machinery may hold potential. Three general ways of improving synaptic function as therapeutic strategies may depend on: 1) pharmacology; 2) genetics and 3) mental exercise. For instance, Metabotropic glutamate receptors (mGluRs) on the dendritic spines are upregulated in FXS, as well as other disorders. So, a pharmacological means of downregulating the mGluRs could be a target for therapeutic intervention. Gene therapy is also under development. In terms of mental exercise, environmental enrichment can promote neuronal activation and experience-dependent plasticity through sensory stimulation. Studies suggest that enhanced stimulation of sensory pathways (e.g. visual, motor, cognitive, somatosensory, etc.) may help to slow down or prevent cognitive decline.



Environmental enrichment promotes maintenance of sensory and cognitive pathways
Nithianantharajah & Hannan, 2006 Nat Rev Neurosci

Conclusion

The morphology of small neuronal processes like dendritic spines affects behavior in physiological and pathological conditions. In fact, we are only beginning to understand how experience and external insults modify dendritic spine behavior. There are many pending questions about the molecules, cells and neural circuits involved in determining normal development and maturation, and about precisely what falters in psychiatric disorders. For instance, we are only at the tip of the iceberg when it comes to addressing the very general and basic question of how learning and memories are established and can be retrieved later on in life. Moreover at the molecular level, we are just beginning to find common synaptic pathways (RNA subsets, RNA binding proteins) affected in different neurodevelopmental and psychiatric disorders. To Dr. Bagni, unlocking the secrets of how these molecular pathways interact at the single protein level is really key to our understanding the pathophysiology of these devastating illnesses, and is essential to finding practical targets for drug therapy.

Intervention on the Synapses to Treat Neurological and Psychiatric Disorders

Dr. Robert Harvey, University College of London, London, UK

Over the last 15 years, major advances have been made in understanding how synapses function. Genetic screening is now being used as a diagnostic tool. As a result, novel neurological disease genes encoding synaptic proteins are being identified from patients with neurological or psychiatric disorders. Based on the identification disease genes, some clinical disorders are now being considered as a matter of the synapses being sick, or as ‘synaptophathies.’ Research at the junction between genetics,

synaptic research and pharmacology has now taken a turn toward disease- and clinically-oriented applications. The goal is to intervene at the synaptic level to treat neurological and psychiatric disorders. In this talk, Dr. Robert Harvey focused first on a rare disorder, the Startle Response Disorder (Hyperekplexia) and then on a set of more common disorders, the encephalic epilepsies. Throughout the talk, Dr. Harvey laid out Ten Lessons about Synaptopathies, as a guideline for future research.

The Startle Response Disorder (Hyperekplexia)

With only a few hundred cases in the world, startle disease has greatly been a neglected disease. However despite being a rare disorder, cases are being reported all the time. In fact the more they publish on it, the more cases they get reported on it. Hyperekplexia is characterized by excessive startle response to sounds or visual stimuli. A diagnostic test taps the baby on the nose and normally the baby throws its arms up in the air, but in startle disease the baby responds with severe muscle stiffness, which can even lead to a halt in breathing and death. Any stimulus (e.g. loud noise, someone walking on the side of the baby, even giving the baby a bath) can trigger one of these episodes. Fortunately there are treatments. One is the Vigivano manoeuvre that consists of actually bending the baby in order to break the seizure. From a clinical perspective, it is unclear how the manoeuvre works, but it does. Benzobenzapines (e.g. Clonazepam) are the second form of treatment and work well at very low doses.

Sequencing revealed that the disease was mapped to the human glycine receptor α1 subunit gene (GLRA1) and later mutations were also identified in the genes encoding the glycine receptor β subunit (GLRB) and the presynaptic Na(+)/Cl(-)-dependent glycine transporter GlyT2 (SLC6A5). The glycine receptor gene has four α-helical membrane-spanning domains, the second of which forms an integral ion channel. This ion channel is upregulated in the brainstem and spinal cord. Dominant mutations were found affecting the glycine receptor α1 subunit. These findings came as a surprise since the disease was initially believed to arise by a completely unrelated mechanism.

Lesson #1: Pharmacology is misleading. Being able to treat a disease doesn't mean you understand it

A cluster of dominant mutations in the second membrane-spanning domains of the associated loops was found (Collab. Mark Rees, Swansea U, Wales). They uncouple ligand binding from channel gating. So these are effectively loss of function mutations. More recently, recessive mutations were found, which demonstrate trafficking defects or loss of the ability to bind the glycine agonist. Despite having a disease gene and mechanism, about 2/3 of cases remain unresolved.

There are many different glycine receptors: alpha 2 and alpha 3 subunits are involved in neurodevelopment. Alpha 3 is involved in rhythmic breathing and inflammatory pain. Many more labs were in fact testing for the alpha mutations than for beta mutations. Through research in the lab, Dr. Harvey's group found that glycine receptor beta subunit was less sensitive if present in in the N2 domain of the surrounding loops, in comparison with the alpha1 subunit. This indicated that the subunits in the receptors are also not equivalent. Recent sequencing has revealed cases *de novo* mutations, with incomplete incomplete dominance, leading to mild startle in the parents.

Lesson #2: Careful comprehensive analysis is needed in genetics

In the case of the beta subunit, the mutation inserted a charged amino acid in the chloride channel. It had no effect on the glycine-based response relationships but did produce cell surface trafficking; this particular receptor also gave spontaneous gating. Therefore too much chloride gets into the cell and in parallel there is also a trafficking defect.

Lesson #3: Mutations can have multiple effects

The trafficking defect was found to be the more important in neurons because it prevented the receptor from reaching the synaptic site, thus making the spontaneous gating defect less crucial.

A study in knockout mice for the GlyT2 gene conduct in Dr. Harvey's lab demonstrated rigid muscle tone, a severely impaired righting reflex, but no signs an evoked startle, as is the case in humans affected by a mutation in this gene. Moreover the mice died prematurely at the second postnatal week, whereas in most of the human cases that undergo clinical intervention, the patients survive.

Lesson #4: Careful phenotyping is important when you are looking at a disease

Dr. Harvey's group characterized a number of glyT2 mutations: initial screening resulted in about six cases with missense or frame-shift mutations. Most of these were loss of function mutations, which means the glycine is not recycled, and the presynaptic terminal consequently runs out of glycine. Furthermore, they

sorted out a dominant mutation in 18 patients form Spain that forms cysteine crosslinks in the extracellular domain of the receptor.
So, again, most of the mutations disrupt either glyT2 trafficking or Na+ or Cl- binding. Interestingly they also found high rates of neonatal apneas, learning difficulties and developmental delay in these patients. And we think it's because they are losing all releasable glycine. So the other subtypes of glycine receptor contain the alpha2 and alpha3 that we know are involved in neuronal migration and breathing is also affected so you get an extended phenotype. In order to make a connection between the genes, proteins, and function, Dr. Harvey's group devised several assays for glyT: transfection of glyT2 in a HEK cell, followed by incubation with tritylated glycine. In the wild type form, uptake of the label occurred, but not with most of the mutants.

Lesson #5: Trust your functional assays, as they may reveal relevant information for the patient

How are benzobenzapines effective in startle disease? The synapse is not as simple as we think it is, with a mix of glycine and gaba receptors. Even though the glycinergic system is broken, we can actually get around it by enhancing the function of the GABAa receptors, which are in the same synapse.

Lessons #6 & #7: Synapses are complex, which can be a beneficial if one can bypass a broken pathway with another signaling system.

Other clinical interventions may be possible. The research studies indicate that patients who have glycine b mutations, glyT2 mutations, have learning difficulties and developmental issues. With a definitive genetic diagnosis, the clinicians can train the parents about their child's disease and give equipment to monitor heartrate and breathing.

Encephalic epilepsies

Excitatory ionotropic glutamate N-methyl-D-aspartate (NMDA) receptors can exist in multiple forms:
di-heteromeric receptors, that form tetramers with GluN1 GluN2A,b,c or d. Tri-heteromeric NMDA receptors that can contain more than one NR2 subunit. They display a stereotyped structure, with amino-terminal domain (ATD), which specifies subunit assembly (involved in receptor assembly), S1 and S2 form the ligand-binding domain, re-entrant pore-forming and transmembrane spanning domains; and the C terminal domain which mediates the location of synapses and trafficking with the PDZ domain binding motif.
Given the role of NMDA receptors in learning and memory, it came as a surprise that is was involved in epilepsy. GRIN2A however plays a role in idiopathic focal epilepsies. These are a very common group of childhood seizure disorders ranging from self-limiting seizure epilepsy to very severe epileptic encephalopathies where you start to get neuronal death.
Why are these focal epilepsies? They are characterized by short, slow waves originating in one area of the brain, often the part of the brain involved in speech production. Many of these disorders have acquired aphasia or loss of speech. Often intractable childhood disorders giving rise to frequent seizures, loss of speech, a devastating set of disorders.
So what do the different studies tell us? Two studies found a huge number of different types of epilepsies with variations of mutations in GRIN2A: atypical rolandic epilepsy (also benign epilepsy with centrotemporal spikes, BECTS), epilepsy aphasia syndrome, atypical benign partial epilepsy (ABPE), epileptic encephalopathy with continuous spike and waves during slow-wave sleep (CSWS), Landau-Kleffner syndrome (LKS). These five different types of epilepsies were associated with GRIN2A mutations. Dr. Harvey's study suggested that *de novo* mutations causing other idiopathic focal epilespies were also responsible, one of the most common forms of childhood epilepsy. It is therefore very interesting to learn that the NMDA receptor is involved.
GRIN2A KO mice have existed for a long time, showing impaired hippocampal plasticity, defects in spatial navigation and learning. However they did not develop spontaneous seizures. How does loss of an excitatory receptor lead to epilepsy, associated with more excitation? Several of these mutations appeared to cause gain of function. For instance, in one publication, a missense mutation in the S1 domain, which is a ligand-binding domain led to an increase in the duration of the ion's open time, letting more ions in the cell. However, there was also some loss of function mutations. In fact they found large deletions, stop codons, frameshift mutations. So one of the big questions was: which of these mutations?

How could these cause disease? Which of them are loss or gain of function? A huge amount of work still needs to be done here. Some have altered closed-state durations; some have a loss of voltage-sensitive magnesium block. It is an important role for coincidence detectors.
Dr. Harvey's group recently identified mutations in another gene GRIN2B in epileptic encephalopathies, causing West syndrome—characterized by infantile spasms and developmental regression, a strange EEG pattern. But in fact variants in this gene have been reported in a variety of disorders, such Autism Spectrum disorders, schizophrenia, focal epilepsy, intellectual disability. The majority have no functional studies associated with them.
Early data suggests that GRIN2B variants are loss of function or gain of function or some seem to have no discernable effects whatsoever.
Lesson #8: Expect the unexpected. A few years ago, NMDA receptor dysfunction as a cause of epilepsy came as a surprise.

Lesson #9 : Not all the variants that are identified in sequencing studies are disease causing.
It's possible that gain of function causes West syndrome in epilepsy and loss of function causes a different disease phenotype like autism or schizophrenia.
Given the NMDA receptor GRIN2B mutations, the Harvey group thought of using a drug called Memantine, as it is an uncompetitive NMDA R antagonist. It seems to work somewhere in the channel binding and has a strong voltage dependency but a very fast off and on rate. So it has also been used in autism and is well tolerated in children. When they expressed one of these mutations, there was a loss of the voltage dependent magnesium blockage. Is this drug still effective on the receptor? The mutation might disrupt the drug's binding site. Memantine however is not perfect because ideally the drug would target GluN2A or GluN2B specifically, whereas this compound targets both and some other ones as well.
Lesson #10: Drug discovery is a work in progress but sometimes very rapid gains can be made by repurposing established drugs that are already clinically approved.
For future research and therapies, Dr. Harvey pointed out that the ERANET-NEURON II call was a real opportunity brings basic research to the clinical interface. The projects with real-world and clinical relevance need better chances of funding. But this is going to require scientists and clinicians to talk to each other. Dr. Harvey mentioned that structures should be developed for treating synaptopathies. We need basic neuroscience research to understand the causes of the disease. It is also important to not develop new therapies when good ones already exist. Rapid gains can be made in drug discovery with repurposing. This means using established drugs that have perhaps not been used for particular clinical applications. There is also a need for better animal models. Dr. Harvey also advocated zebrafish as a tool for disease gene screening. Zebrafish comprise a model to test the mechanisms and treatments of disease, with drugs to try to recover phenotypes. There is a need to make use of the results from next generation gene sequencing, but also functional assays. Especially with electrophysiology, Dr. Harvey advocated high-throughput electrophysiology from networks of neurons. Great gains in personalized medicine can thus be made.

Annex I

List of Participants

Speakers at the scientific workshop 'Synaptopathies'

1. **Prof. Eckart Gundelfinger**, Leibniz Institute for Neurobiology, Magdeburg, Germany
2. **Prof. Daniel Choquet**, University of Bordeaux, France
3. **Prof. Pierrette Gaudreau**, University of Montréal, Canada
4. **Prof. Monica Di Luca**, Milan University, Italy
5. **Prof. Claudia Bagni**, University of Leuven, Belgium
6. **Prof. Robert Harvey**, University College London, UK

NEURON II SAB members and additional experts

1. **Prof. Celso Arango** (Universidad Complutense, Madrid, Spain)
2. **Prof. Vania Broccoli** (San Raffaele Scientific Institute, Milan, Italy)
3. **Prof. Francois Berger** (University of Grenoble, France)
4. **Prof. Eero Castren** (University of Helsinki, Finland)
5. **Prof. Joab Chapman** (Sheba Medical Center, Tel Aviv University, Israel),
6. **Prof. Monica Di Luca**, (Milan University, Italy)
7. **Prof. Martin Dichgans** (Ludwig-Maximilians-University Munich, Germany)
8. **Prof. Isabel Farinas** (University of Valencia , Spain)
9. **Prof. Christophe Mulle** (CNRS, Institut Francois Magendie, France)
10. **Prof. Andreas Meyer-Lindenberg** (University of Heidelberg, Germany)
11. **Prof. Robin Ali** (University College London, UK)
12. **Prof. Fabrizio Tagliavini** (Istituto Nazionale Neurologico Carlo Besta, Milan, Italy)
13. **Prof. Moussa Youdim** (Rappaport Institute, Haifa, Israel)
14. **Dr. Ana-Maria Zagrean** (Carol Davila University of Medicine and Pharmacy, Bucharest, Romania)

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