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## How to make a KO or Tg Mouse

* Construct delivered to embryonic stem cells.
* Embryonic stem cells are selected according to an antibiotic resistance which tells us which cells are carrying the modification that we want to introduce into the animal.
* Selected stem cells are implemented into an embryo that blastocyses.
* Then they are transferred to a female host.
* The pups that are born are chimera, some cells have the gene modification and others not.
* Then by crossing chimera with WT we know if gene modification passes to next generation.

## Inserting Gene with homologous recombination

* When the gene is flanked by two DNA regions that have the same sequences to other sequences present in the genome, the sequence in between these two flanking sequences could be exchanged. Cellular enzymes that cut, paste and put together the DNA strands but requires sequence homology in flanking regions. In between the two regions we add a selection marker, usually resistant to neomycin.
* A negative selection marker is added for TK. The TK gene is a suicide gene which converts ganciclovir into a lethal gene.
* When the targeted gene is correctly replaced, the TK gene is not inserted into chromosome along with the NeoR gene, so the resultant cells are resistant to both neomycin and ganciclovir.
* For a KI model, neomycin is replaced with the gene to be inserted and replace the target sequence. And the flanking sequences are random sequences. The insertion of the new gene can occur randomly: transgene could be inserted into a vital sequence.
* Also, for KI model, the transgene can be inserted into DNA silent regions and not translated so there is not enough expression of the transgene.
* Another issue with transgene could be overexpressed and the phenotype of the model could be influenced.

## How to make a mouse model with CRISPR/Cas9

* We need CRISPR/Cas9 and an oligo element used as template to insert or delete sequences in order to KO target gene. These changes are directly made in the embryo not in the embryonic stem cells.
* For Ki or Tg mice, we need to add in addition of CRISPR/Cas9 a template plasmid carrying the gene which is going to replace for KI or to be added for Tg mice: less time consuming, cheaper and more efficient.
* Conditional KO, KI, Tg model
* gene modification is not always present but induced after stimulation, useful to be activated at any time and closer to what is observed in many disorders which occur later in life.
* Conditional KO mice
  + Needs 2 models
  + 1 mouse line contains a vector containing a Cre recombinase under control of tissue-specific or inducible Cre. This line represents on switch for gene modification.
  + Second line contains a target vector which has specific sites called LoxP sites recognized by Cre recombinase inserted on either side of the gene to delete. Cre recombinase recognizes Loxp sites and cuts sequences within the two sites.
  + When the two mice are crossed: Cre is expressed under stimuli and the recombination occurs at Loxp sites and will delete the gene in between the 2 Loxp sites: KO only on stimulation.
* Conditional KI mice

The construct uses different types of Loxp sites and the Cre recombinase is used to remove the gene to be deleted and replace with the new gene.

* Conditional Tg mice

The 2 Loxp are engineered to flank a stop sequence followed by the gene to add. When Cre recombinase is used the stop sequence is removed.

# Advances in Microscopy

## Small molecules Probes for Bioimaging

* Possibility to isolate and visualize, and switch on-off specific neural circuits under line. natural behavior or cellular processes as they happen in-vivo.
* Small molecules are small fluorescent compounds that can bind specifically to a target. These bindings affect their properties and their properties will change their fluorescent spectrum or their affinity with the target.
* Ca2+: we can see neurons firing
* K+, Na2+: important to record AP
* To visualize more complex cellular processes: like oxidative stress, apoptosis and organelle viability.
* JC-1: allows to visualize membrane potential in-vivo.
* Other compounds confirm the existence of mitochondria, lysosomes (lisotracker), organelles (ER tracker)
* Possibility to use non ratiometric or ratiometric probes:
* **Non ratiometric**: can register the presence of a substrate but cannot qualify the binding in vivo.For ex. Lysotracker can visualize where the lysosome is in the cell but cannot determine how many there are or if they are viable (same for organelle tracker).
* **Ratiometric**: can measure two different wavelengths ratio of absorbance / ratio of emission.
* Non-toxic in-vivo
* Able to cross the cell membrane
* To measure Ca2+ in cells: 2 Ca2+ small molecule probes: Fura-2 (ratiometric) and Fluo-3 (non ratiometric).
* Range of concentration of Ca2+ in cell varies from cell to cell or organelle to organeel. Goes from 50nMolar to 50 microMolar.
* Ratio: bound Ca2+ / free Ca2+ varies from cell to cell.
* In ER: 10/1.
* The probes themselves can affect calcium kinetics.
* Choose probe with right affinity by knowing what is the concentration of Ca2+ to be registered.
* Not possible to control localization of the dye: impossible to target specific organelles, specific domains, specific subsets of cells.
* Sometimes loading is difficult.
* Can leak

# Genetically Coded Optical Indicators

* Fluorescent probes developed using fusion proteins.
* Measure selectively a target and it is possible because it can be expressed in a cell or cell tissue in a specific manner.
* Can be used for extended experiments.
* Needs to be expressed in cell tissues, so it needs to perform a transgenic expression using a vector containing GEI to be injected directly in the tissue to be observed (viral expression) or in utero expression.
* In utero electroporation (IUE) is a technique developed to introduce plasmid DNA into embryonic mouse brains, while the animals are still alive in the uterus. Basically, by electroporating a DNA construct into a subpopulation of progenitor cells in the ventricular zone of embryonic brain, the progenitor cells carrying the DNA will undergo neurogenesis, migration, and final differentiation to become mature neurons positioned in distinct cortical layers according to their birth date. In addition, by controlling the direction of electroporation, a specific cortical area can be targeted. Thus, in utero electroporation allows gene modification in a specific cortical layer in a specific cortical area. Viral expression with utero electroporation does not allow to develop stable genetic models because GEI is not expressed in germline. Utero electroporation performs in-vivo genetic manipulation of specific cell types of different brain locations while the animal is alive in the uterus.
* GEI based on GFP promoters and variants (YFP).
* FRET technique uses two fluorescent proteins: Donor and acceptor which absorbs energy from donor. The second protein emits energy only when the two proteins are in close proximity.
* Synaptic vesicle release is fundamental in synaptic signaling and can be detected by genetically encoded pH indicators (upon protonation or membrane fusion DeltapH: 5.5 to 7,7.5). When the fusion of the vesicle happens the GEPI becomes deprotonated and fluorescent. So, when synaptic transmission occurs we can see neurons firing. phVorin.
* Neurotransmitter indicator GETI to visualize glutamate which is primary neurotransmitter in CNS. **GETIiGluSSnFR** uses permutated GFP: **cpGFP** to bind to glutamate transporter **GPt1**. cpGFP deprotonates when Glu binds to Glt1 enabling the visualization of post-synaptic activation.
* **CAMGAROO**: a protein obtained by fusing YFP with calmodulin binding domain which is the protein binding Ca2+.

# Molecular Basis of Optogenetics

* Neurons are first genetically engineered to express light sensitive proteins called opsins. When neurons are illuminated with correct light frequency they are transiently activated or inhibited or their signaling is modulated depending on the opsin expressed.
* Genetically targeted optical control of neural activity.
* Cell type specific expressions:
* Creation of Tg animals
* Use of viral-vectors to express opsins
* Electroporation
* Millisecond-timescale
* Opsins are proteins which modify the activity of the cells in which they are expressed when the cell is exposed to the light.
  + Tight control of excitatory or inhibitory synaptic transmission
  + They can trigger a single or multiple APs
  + They can suppress neural activity
  + They can modify signaling pathways with ms control of timing event.
* ChannelRhodopsin2 is gated-light sensitive cation channel. The transfection could be done with a lentiviral gene expression system.
* When retinol binds to opsin, the retinol becomes light sensitive.
* Optogenetic actuators allow to biomedically change specific cells or protein functions: control of gene expression.
* Luciferase is protein that can degrade specific substrate and emits light. When its promoter is under control of channelrhodopsin we can induce luciferase transcription by illuminating the cell. Luciferase gene is under promoter of a sequence that is recognized by Gal binding domain protein. The Gal domain protein can be fused to channelrhodopsin. When the cell ix illuminated with light, the channelrhodopsin is activated which activates Gal domain protein which binds and activates transcription of luciferase.
* Expressing opsin VTA region of the mouse.

# Introduction to neurodegeneration

* Basic mechanisms are multifactorial: interaction between genetic and environment and endogenous factors. Aging: multi-risk factor.
* Neurodegenerative diseases share common features:
  + Aggregation of cytosolic or nuclear proteins, aberrant aggregation of cell misfolded proteins.
  + Formation of high order inclusion of fibrils.
  + Normal native proteins can become gradually misfolded and self-aggregate into insoluble polymers, the cells acquire a define fibril structure known as amyloid (type of specific structure for a protein).
  + Extra-cellular protein aggregate (AD) and intra-cellular protein aggregate (PD).
  + Extra-cellular aggregate affect neurons.

The Ubiquitin/Proteasome System (UPS) is a highly regulated mechanism of intracellular protein degradation and turnover. ... The UPS participates in a wide array of biological functions such as antigen presentation, regulation of gene transcription and the cell cycle, and activation of NF-κB.

## Common cellular pathways in neurodegeneration

1. Protein quality control (HP, UPS)
2. Autophagy-lysosome pathway
3. Mitochondrial homeostasis: mitochondrial dysfunctions result in oxidative stress and free radical formation, synapse impairment, ER Golgi stress, defective Ca2+ signaling, impairment in axonal transport
4. Protein seeding and propagation
5. Synaptic toxicity and network dysfunction

**Dying back theory**: Deficiencies can be localized in ALS and PD. These damages initiate at axon synaptic terminal and move back to cell.

Protein aggregate ⬄ dysfunctions: collective dysfunction of many cell mechanisms

**Excitotoxicity**: excessive activation of receptors of excitatory amino acids.

**Glutamate** is the most abundant free amino acid in the brain and is at the crossroad between multiple metabolic pathways.

Excess of glutamate results in Ca2+ overload which can lead to activation of enzymes that degrade proteins, membrane which causes neuronal death

In AD, neuroinflammation is probably the primary cause. Memantine is used to block Excitotoxicity. Formation of ABeta plaques is directly or indirectly responsible excessive activation of NMDA receptors for glutamate.

## Other common systemic deficits in neurodegeneration: accumulation of iron

* Iron is used in mitochondria respiration.
* In CNS it is present in many cell types including: neurons, astrocytes and microglia.
* Iron participates in electron transfer by switching to a different state: Fe2+ => Fe3+.
* Iron is present in regions such as beta ganglia.
* Different levels of ROS (Reactive Oxygen Species) are produced during these types of reaction.

# Alzheimer Disease – plaques – tangles

* Accumulation of senile plaques and neurofibrillary tangles in the brain
* Atrophy of cerebral cortex, hippocampus
* Ventricle expansion

Amyloid precursor protein (APP) is processed sequentially by the β-site APP cleaving enzyme and γ-secretase to generate amyloid β (Aβ) peptides, one of the hallmarks of Alzheimer's disease. The intracellular location of Aβ production—endosomes or the trans-Golgi network (TGN)—remains uncertain.

APP eventually dies out and needs to be recycled and dispensed of. APP is broken down in 2 pathways:

* Non-amyloidogenic pathway: beta-secretase and gamma-secretase which produce amyloid beta monomers.
* Amyloidogenic pathway: beta-secretase and gamma-secretase.
* AD: increase of beta amyloid production and decrease of beta amyloid breakdown.
* Beta amyloid monomers => amyloid beta oligomers => aggregate oligomers => senile plaques which block neurotransmitters in synapses.
* Microtubule act as highway to transport the signal back and forth between soma and axon terminal. Tau protein stabilize the microtubule. In AD it detaches from microtubule to form neurofibrillary tangles.
* Decrease in CHAT (Choline Acetyl Transferase) is correlated with the number of senile plaques and AD severity.

# Alzheimer’s disease: beyond the APP hypothesis

**Neuro fibrillary tangles**: paired ligaments made by hyperphosphorylated tau, AB plaques aggregated amino beta peptides

**Cholinergic neurons**: specifically affected by tangles and plaques. In AD there is a decrease in cholinergic transmission.

**Amyloid cascade hypothesis**: there is no evidence for it.

## Neuroinflammation in AD

In AD, there is a persistent activation of immune cells which increase tissue infiltration of microglia. Astrocyte and microglia cells are the main types of cells in inflammation response in the CNS. They have the capability to phagocyte toxic product by releasing cytotoxic factors and neurotoxic agents to wash the CN damages.

**Astrocytes**: are glial cells which play an active role in synaptic and neuro genesis. Their activation is important in beta clearance and degradation.

**Microglia cells**: represent 10% of CNS cells and play an important role in neuronal genesis, plasticity and regeneration.

In AD, the activation of astrocytes become persistent and can promote ABeta dispositions by increase of creation of beta-secretase.

The ROS are produced by cells that are involved in the host-defense response, such as polymorphonuclear neutrophils (PMNs) and promote endothelial dysfunction by oxidation of crucial cellular signaling proteins such as tyrosine phosphatases. The ROS act as both a signaling molecule and a mediator of inflammation.

## AD Cascade and Neuroinflammation

Neuroinflammation can occur early in the disease and that can cause neuronal death.

## Cerebral Vascular Angiopathy in AD

* Brain endothelial cells, pericytes, vascular smooth muscle cells, glia, neurons control BBB permeability, blood flow and composition of the environment.
* Deregulated transport of metabolites, passage of neurotoxic and pro-inflammatory molecules through BBB.
* Excessive accumulation of ABeta in the brain parenchyma and peripheral vascular regions damage micro-vessels compromising BBB which leads to disregulated transport of metabolites, possible invasion of pathogens, major infiltration of glia cells, and increase of neuroinflammation.

## Visualizing AD

* Traces and neurological assessments using:
* Amyloid PER imaging
* PIB-PET
* Tau PET imaging

PET imaging cerebral metabolic of glucose uptake using deoxidized glucose FDG to detect functional changes in AD. Reduction of FDG uptake: occurs especially in parietal and posterior cingulate cortex of AD patients.

* CAT scan or MRI: to detect brain atrophy, cognitive impairment, structural abnormalities, presence and extent of cerebral vascular diseases

## Systemic AD

* Connection of ABeta and anti-micro-biopeptides.
* Infectious areas are nasal and buccal cavities.
* AD connected to a specific microbe.
* **Porphyromonas Gingivalis**: can secret a protease gingipain a pathogen responsible for gum disease, is present in AD patients.

# Pharmacology – Drugs for Alzheimer’s disease

1. Cholinesterase inhibitors
2. NMDA receptor antagonists

* Agents preventing beta-amyloid fragments from clumping into plaques by targeting 2 enzymes: beta-secretase and gamma-secretase which cut amyloid precursor protein.
* Antibodies that bind to beta-amyloid and enhance clearance from the brain
* Tau protein:
* Clearing pathological tau protein
* Compounds that prevent tau aggregation or dissolve existing aggregates as well compounds that inhibit microtubule disassembly

# Molecular Basis of ALS

Frontal Temporal Dementia (FTD): frontal and temporal lobes of the brain experience atrophy and neuronal death similar to ALS.

15% of FTD patients develop MN dysfunctions

50% of ALS patients develop FTD

Multitude pathological factors: development and progression of ALS, genetic and phenotypic variations between individuals, make difficult to reach conclusions.

Express as:

* Disturbances in RNA metabolisms
* Impaired protein homeostasis
* Nuclear cytoplasmic transport defect
* Impaired DNA repair
* Excitotoxicity

**Research focus on RNA deregulation**:

* 2 RNA binding proteins have been identifies as causing variations in the disease: TDP 43 and FUS (involved in splicing, transcription and transport).
* SOD1: enzyme to convert dangerous O2 radicals in less dangerous ones.

**Stress granules**: transient RNA protein complex formed under cellular stress, able to sequester messengers RNA and prevent translation. They facilitate cell survival by translation of non-essential transcripts when cells are under stress. They have been associated with ALS.

**TDP43**

* Main component of inclusions in ALS patients, pathological hallmark of ALS.
* Present on neuronal cytoplasmic inclusion (NCI).
* Formulation inclusion bodies leads to cellular dysfunction.
* Nuclear depletion of the protein induces widespread deregulation of RNA messenger metabolisms.
* Thus, tight control regulation of TDP43 is essential. Normally expression of TDP43 is auto-regulated.

**C90RF72**

* Implicated in RNA metabolism and protein homeostasis.
* Loss or gain function of C90RF72 are both implicated in the mechanism of pathogenesis of ALS.
* Hexanucleotide repeat expansion in mutated C90RF72 gene and only such expansion known in Huntington disease.
* Gain of function leads to RNA being misprocessed and can accumulate in stress granules.
* Messengers of C90RF72 form discrete nuclear structures referred as **RNA fossils**.
* C90RF72 mice

## Neuroinflammation in ALS

* Examination of post-mortem tissues or animal models (Tg mice for SOD1)
* Microglia activation of human cortex similar to tissue injury and sporadic ALS: proliferation activation characterized by enlarged ramification.

**Reactive astrocytes present in ALS**

Mouse with mutated form of SOD2 (Tg SOD1G93 mice) labeling with antibodies s which recognize only activated astrocytes

**Astrocyte and microglial**

* Support activity of MNs
* Excitatory amino acid transporter (EAAT)-2 is one of the major glutamate transporters expressed predominantly in astroglia cells and is responsible for 90% of total glutamate uptake. Glutamate transporters tightly regulate glutamate concentration in the synaptic cleft. Dysfunction of EAAT2 and accumulation of excessive extracellular glutamate has been implicated in the development of several neurodegenerative diseases including Alzheimer’s disease, Huntington’s disease, and amyotrophic lateral sclerosis.

Loss of glutamate => loss of EAAT2

* + Leads to ecotoxicity in motor cortex or anterior horn of spinal cord identified as pathological mechanism in ALS
  + Impair myelination
  + Affect MNs

Drugs:

* Riluzole
* Edaravone

**Prion-like protein** spreading and transmission of aggregates between cells have also been demonstrated for other proteins associated with Alzheimer disease and Parkinson disease.

Pathological proteins in neurodegenerative diseases adopt prion-like mechanisms and spread across the brain along anatomically connected network

# Parkinson Disease

Disease where dopamine neurons in the susbtantia nigra of the brain undergo degeneration.

The substantia nigra can be split into 2 sub-regions:

* **The pars reticulata:** receives signal from another part of the basal ganglia: the **striatum**, which is a term for the caudate and putamen put together and it relays messages to the thalamus via neurons rich in the neurotransmitter GABA (gamma aminobutyric acid).
* **The pars compacta**: part of the substantia nigra affected in PD. The pars compacta send messages to the striatum via the nigrostriatal pathway which helps stimulate the cerebral cortex to initiate movement. When substantia nigra pars compacta die: individual may be in hypokinetic (loss of movement state) commonly seen in PD.
* Dysfunction in dopaminergic signaling in other parts of the brain beyond substantia nigra, for ex. In prefrontal cortex.
* Distinctive pathology is clump of misfolded proteins: Lewy bodies. These proteins can form small repeated units called oligomers or longer fibrils.
* Unwanted proteins are cleared by different types of protein degrading machinery: proteasome and autophagosome. Some evidence points that these systems can be overwhelmed by misfolded alpha-synuclein proteins.
* PD is also linked to problems with mitochondria which provide cells with energy to perform vital functions: this process can be impaired. Autophagosome impairment induces damaged or worn-out mitochondria.
* As dopamine neurons are lost: microglia take up resulting cellular debris triggering immune response and release of inflammatory cytokines which activate neighboring microglia and astrocytes resulting in injured neurons.

**Potential treatments**

* Enhancing the clearance of abnormal proteins and blocking their transmission.
* Improving the function of mitochondria
* Targeting the neuroinflammation

**Drugs**

* Peripheral Dopa Decarboxylase, side effect: arythmias
* Levodopo administered with carbidopa: a dopamine decarboxylase inhibitor.

# An update on PD

* The clinical aspect of PD is mainly based on neurological exam thru presentation of 4 cardinal motor symptoms.
* Parkinsonism: overlap with other parkinsonisms such as:
  + MSA: Multiple system Atrophy
  + PSP: Progressive Supranuclear Palsy
* No neuroimaging is specifically recommended as a routine in clinical practice for PD.

**CAT Scan**

Could be used for identification of structurally lesions associated with other forms of parkinsonismss or underlying conditions including vascular pathology and neoplasm.

**Structural RMI**

Measures distribution and degree of brain atrophy

**PET Imaging with FDG**

* Shows regional brain metabolism.
* Can be informative for PD diagnosis which is characterized by an increase in basal ganglia, pons cerebellum, with concurrent reductions of glucose metabolisms pre-motor and posterior, parietal cortex.
* Can be used to differentiate PD from MSA and PSP.

**SPECT Imaging**

Nuclear imaging scan that integrates computed tomography in a radioactive tracer

DAT-SPECT for synaptic dopaminergic functions is able to identify DAT (Dopamine Transport), helps to visualize dopamine transporter levels in the brain

**DOPA DDC PET**

Shows reduction of such activities in PD patients.

Definite diagnosis of PD is only possible post-mortem with histological examination of alpha-synuclein inclusions and dopaminergic neuronal loss.

19 different genes have been associated with genetic forms of PD. But this account only for 5% of all PD cases. They are risk-genes associated with higher probability of developing PD.

## Gut-brain axis in PD

* Presence of alpha-synuclein inclusions in the enteric system
* Alpha-synuclein is a prion-like protein that can propagate and spread from neurons to neurons
* It could be the result of an infectious agent
* Certain bacteria can secrete amyloid proteins which can function as prions and can initiate directly cascade of events which culminate in alpha-synuclein misfolding. These proteins can navigate thru vagal innervation to spinal cord and brain stem.

# By passing BB: Treating PD

**BBB**

* Protects brain from bacteria and other diseases
* Prevents large drugs including proteins and gene therapies from getting into the brain when taking orally or injected into blood stream
* Invasive for elderly and quite risky: drugs don’t distribute well into the brain (remain at injection site or only penetrate the superficial layer of the brain)
* Do not reach deeper regions usually the most affected

**Intranasal delivery**

* 2 pathways directly connected the nose to the brain, which is the result of 2 main nerves ending inside the nose and that reach all the way up into the brain.
* 1 hour to reach the brain.
* Non-invasive.
* Easily repeatable.
* Directly injected into the brain: prevents side-effect in unfaceted body organs.

**GDNF**: protein that protects neurons.

# Models of Neurodegeneration

**Genetic animal models**

K-I, K-O, transgenesis gene associated with the disease

**Toxin-induced models**

Toxin which is associated to the loss of a particular class of neurons. The toxin can induce an accumulation of a selected type of inclusions.

**Amyloid-seeded models**

* Amyloid position is induced by exogenous administration of protein aggregates obtained from in-vitro fertilization from genetic animals or brain tissues of sick patients.
* Based on prion-like protein to propagate.
* Mostly used for alpha-synuclein and tau.
* Induces extra-cellular formation of protein aggregate.
* Models work well with primary neurons and mice.

**iPSCs**

* Obtained from patients.
* Culture of isolated cells.
* It is very informative since it provides. A window on toxic process happening in real-time.

**AD**

Tg 2576: combines combination of ABeta/Tau together.

Tg Mice obtained with antibody against NGF which induces in adult mice loss of cholinergic neurons in fore brain and hippocampus with memory loss.

**ALS**

Models only informative about variant. They cannot be used to make general conclusions.

**PD**

* Acute models using exogenous accumulation of toxins such as MPTP, 6-OHDA to target specifically dopaminergic neurons.
* Researchers do not always get alpha-synuclein inclusions.
* Injection directly into substantia nigra or striatum and only in one side of the brain using the contralateral side ss control.

**LPS**

* Inclusion of bacteria toxin (LPS)
* Induction of neuroinflammation in the CNS and dopaminergic neuron loss

## Limitations of Neurodegeneration Models

* Primary culture work better than cell lines
* Inclusion formation difficult in cell lines and not reproducible

# Therapeutic Approaches in Neurodegeneration

* No cure since most of cases are sporadic
* But some therapies can improve some symptoms to a certain level

**Cholinesterase inhibitors**

**Antipsychotic drugs**: to treat behavioral and psychological symptoms of dementia

**Analgesic**: drugs for pain

**Anti-inflammatory drugs**

**Brain stimulants**: to stop tremors

**Vaccines** against protein to form of inclusions

**Antibodies** against plaques and inclusions

**Antisense oligonucleotide, siRNA**:

interfere with protein translation of the target allowing the continuous accumulation of proteins.

Working well on hereditary disease like Huntington disease.

Lower levels of alpha-synuclein and tau.

**Overexpression of neurotrophin factors** such as NGF or BNDF to increase neuronal survival

**Recover expression of missing or altered proteins** thru gene therapy such as SMN1

Cell therapy replacement: replace neuronal population using stem cell injection: use prion-like protein behavior to propagate, however there is a good chance that new neuron be absorbed by cytosine protein aggregate.

**Antibody**:

* Decrease protein turnover and clearance of prion like protein.
* Safer than vaccine.

**Aducanumab**: could decrease the number of amyloid plaques by blocking 2 protein interaction.

* Difficult to overcome BBB
* Urgent to find biomarkers for early diagnosis

# Cellular And Molecular Basis of Nerve Regeneration

## Regeneration of the peripheral nerve (PN)

Most of axons fail to regrow after injury in CNS

Axons of PNS can regenerate

* To regenerate axons, need to promote the growth of axonal growth cone
* Requires multiple cell types: neurons, Schwann cells, endothelial cells, macrophages
* Needs local cytoskeleton modifications
* Transport of building blocks from soma to axon
* A retrograde transport of injury signals to tell soma to activate
* Transcription factors to trigger pro-regenerative programs
* Proper mitochondria trafficking
* Epigenetic modifications
* In both proximal and distal segments of the injured axon, the bridge of the cell membrane triggers depolarization and rise in intra-cellular Ca2+ which leads to membrane resealing and cytoskeleton remodeling

Two different processes:

* **Proximal segment undergo regeneration**

Increase of intracellular Ca2+ which induces activation of phosphatase kinase and protease responsible in endoskeleton and formation of growth cone

* **Distal segment undergoes Wallerian degeneration**

Increase of Ca2+ influx leads to activation of SARM1 a major factor of degeneration response. Active SARM1 initiates signaling cascade leading to activation of protease such as **calpain** which start to break down cytoskeleton structure.

## Stages of Regeneration in the PN

Proximal segment navigates into 2 environments:

1. Bridge of new tissue
2. Distal stump: the two nerve stumps are reconnected by a bridge of cells composed of inflammatory cells: Schwann cells, neurons, fibroblast and matrix.

* Schwann cells dedifferentiate to progenitor cells and with tissue macrophage ad inflammatory cells remodel the environment to make it more conducive of axonal growth.
* When Schwann cell merge from both stumps, they encounter fibroblast, the encounter signal in SC result in the formation of cellular cords which transport axons across the bridge. They move along the surface of the polarized blood vessels.
* The blood vessels form in response to hypoxia. The bridge is poly-vascularized and results in the lack of O2 specifically sensed by macrophages which trigger blood vessel formation by secretion of VEGF which stimulates neuro-angiogenesis.
* When the bridge is vascularized: Schwann cells can migrate along the vasculature taking with the regrowing axons into distal stump.
* SSC form the Bands of Bungner which are cords of cell that help:
* To direct the growing axons from original stump to target.
* Clear the axonal and myelin debris.
* Model environment to create a more conducive environment allowing axonal growth.

## Macrophages: important actors in regeneration of PN

* Stimulates vascularization
* Remove cellular axonal and myelin debris
* In ganglia secrete factors important in conditioning neurons toward a generative phenotype acquired by a selected set of genes associated with regeneration: RAG Regenerative Associated Genes.
* (RAGs) have been identified from these studies, the response to injury likely regulates the expression of functionally coordinated and complementary gene groups. For instance, successful regeneration would require the induction of genes that drive the intrinsic growth capacity of neurons, while simultaneously downregulating the genes that convey environmental inhibitory cues.

## Schwann Cells: primary actors in the regeneration in PN

SCs dedifferentiate and proliferate, act as central mediator and orchestrator of regenerative response

* Control breakdown BBB and influx of inflammatory cells
* Clear environment of myelin and axonal debris
* Support axonal growth
* Control macrophage influx

## Signaling Pathways activated during PN regeneration

* RAG pathways are important in the formation of axonal growth cone: ex. activation of cyclic AMP cascade (PKA).
* Rho: molecule important to promote pathways known to inhibit regeneration. Major role in lack of regeneration in CNS.
* Pl3/AKT, RAS/ERK, Roh/ROC

## Specificity of Axonal Regeneration

Activation of RAG pathway and the formation of axonal growth cone is necessary for an efficient regeneration ability. But a successful regeneration depends on a permissive environment. And axonal guidance cues.

Axon guidance cues: axon growing towards the correct target depends on:

* Extra-cellular proteins.
* Presence of neural traffic factors.
* Cytokine.
* Presence of specific adhesion molecules produced by glial cells specific for nerves and muscles.
* Presence of topic factors.

# Nerve regeneration obstacles and differences between PNS and CNS

Obstacles to nerve regeneration in the CNS

* Environment critical determinant of axon regeneration
* PNS: stimulatory – Growth promoting factors
* SCs are the primary glial cells which myelinate peripheral axons and also form a continuous basal lamina.
* CNS neurons do nt upregulate growth associated genes to the extent of PN neurons.
* CNS: inhibitory for axon regrowth = growth inhibitory factors: MAG, Nogo, NgR, OMgp
* CNS: oligodendrocytes myelinate axons but do not produce continuous basal lamina in association with the axons.
* Basal lamina: provides axonal guidance to regenerate axons.
  + Debris clearance is slower also there is a formation of glial scar that will impede the physical elongation.
  + Not permissive environment;
  + Myelin-associated inhibitors, MAI – expressed by oligodendrocytes – not found in PNS
  + (Nogo-A, MAG, OMgp); Only MAG present in PNS myelin but cleared rapidly
  + Astroglial scar: contains molecule inhibitor CSPG which impede axonal growth (chondroitin sulphate proteoglycans, CPSGs);
  + Axon Regenerative Inhibitor Pathways (Rho-A) – inhibits formation of cytoskeleton structures and it is only in CNS
  + Modest upregulation of RAGs

# Experimental Approach to PM Regeneration

**Axonetmesis**

* Demyelination + axon loss.
* Involved surgery.
* Nerve not cut.
* Regeneration process is very fast: difficult to distinguish between different groups.

**Neurotmesis**

* Nerve is cut.
* More difficult surgery.
* Differences between the groups.
* Micro-surgery is needed to reattach the two stumps.

Eventually SCs progress loses support growth phenotype, becoming atrophic if there is a too long distance and is unable to support regeneration

Increase period of time: also stump/ muscle – end target become atrophic

**Acetyl-L-Carnitine (ALCAR):** reducing neuronal death and promoting axonal growth

**Fasudil**

**Graft**

Piece of nerve harvested switched between proximal and distal stump

**Autograft**

Harvested from expendable nerve site and from same patient

**Allograft**

From donors: immune suppression required

**Nerve transfer**: nerves from less important zone or redundant areas to redirect without cut to restore function to a nerve severely damaged.

**Nerve conduit**: bridge gap of a sectioned nerve. Protect nerve from surrounding environment. and prevent the formation of a scar, guide the nerve to distal stump.

# Regeneration in the CNS

CNS neurons after injury have at their end of their proximal stump a retraction bulb. In the retraction bulb, stabilization of the microtubules with concomitant axonal transport do not occur. Microtubule stabilization using **TAXOL**, a compound targeting cytoskeleton, was able to interfere with the formation of retracting bulb and facilitated growth formation in-vivo.

## Development And Regeneration

**Critical period**

When CNS is immature during fetal development, axons in the CNS can regenerate and extensive neurogenesis.

* During development growth cone similar to the ones after injury: growth cone with finger like filaments made of polymerase actin that protrudes from proximal stump.Developing neurons regeneration associated genes (RAG) and proximal stump of growing neurons have rich mitochondria and high level of energy supply.
* On the contrary, after injury in adult CNS, adult neurons express gene that express growth-restricting genes and retraction bulb has disorganized microtubules with accumulating depolarized mitochondria and ATP drops drastically.

## Development and Regeneration: WNT Pathway

* Wnt proteins are expressed in spinal cord grey matter along rostral caudal axis in a concentration gradient that decreases from rostral to caudal.
* Wnt proteins are sensed by two proteins: Ryk and Frizzled which are on the neuronal membrane. The binding leads to deactivation of Ca2+ channels that will increase intracellularly the concentration of Ca2+ leading to an activation of various pathways that leads to a growth cone inhibition.

**Development**

Cortical spinal axons steer away from the brain towards their spinal targets in the white matter by sensing Wnt proteins which are repulsive guidance cues that express rostrally then caudally in the grey matter. Following the decreasing concentration oof Wnt proteins, cortical axons can move away from the brain.

**In adult brain**

Wnt protein not expressed. After adult injury, cortical spinal axons inhibited from sprouting by Wnt proteins expressed at the injury site. Ryk KO enables axons to sprout around spinal cord injury and regenerate.

## Development And Regeneration: other extra cellular clues

**Development**

Extra-cellular matrix secreted by astrocytes, oligodendrocytes and myelin.

Cells of astrocyte linage play a guiding role in axon path finding and circuit formation during development. They secrete extra-cellular matrix proteins: CSPGs that form repulsive barrier for axonal growth.

**Adult injury**

Astrocytes become reactive and direct the formation of a scar at injury site by walling off a core of cells such as macrophages. This is important to mitigate inflammatory damage and this way is neuroprotective. Many scar cells inhibit axonal growth by interacting with tips of axons or by secreting extra cellular matrix molecules such as CSPGs.

**Epothilone B pr taxol treatment**: to stimulate growth cone formation

* Formation of myelin sheets along the axons have been considered potential mediator of the critical period.
* Differences between development and adult injury
* Axon regeneration associated genes are similar but use different promoter which are not active during development.

# Experimental Approach to Nerve Regeneration in CNS

* Legion to optic nerve, spinal cord: methods of CNS injuries
* DRG neurons bifurcate in two branches
* Peripheral branch target to cell body
* Other branch projects in CNS to dorsal column of spinal cord
* Peripheral branch of DRG has greater ability to regenerate compared to central branch resulting in a functional recovery. Central branch does not regenerate.
* Peripheral branch of PNS is in a stimulatory environment compared to inhibitory environment of CNS.
* When central branch injury happens after peripheral lesion: central branch regenerates into and beyond injury site into inhibitory element of spinal cord.

## Conditioning Peripheral Lesion

* Increase of regenerative response of central axons.
* Effect of conditioning relies on a transcription program induced by signals that are transported retrogradely to nucleus.
* Once axons reach its target, intrinsic neuronal growth program shutdown by signals produced by the target itself.
* Conditioning boosts regeneration in optical nerve.

## Models for Spinal Cord Injury

Contusion and compression are most frequent in humans

CSSI rat model: large field cyst cavity formed similar to human injury

## Strategies to improve nerve regeneration in the CNS

* Combinatorial effects of multiple factors
* Stabilization of microtubules and cytoskeleton with compounds clinically used for cancer therapies.
* Epothilone B can cross BBB and exhibits differential effects:

**In neurons**: rapid microtubules polymerization into neural tip promoted axon regeneration

**In fibroblasts**: it prevents microtubules polymerization towards cell edges, inhibiting scar formation.

* Boosted mitochondria functions to improve energy supply,
  + or more active mitochondria transport towards the axonal tip
  + more healthy type of mitochondria to boost ATP supply production
* Boosting of pro-regenerative signals: activating inflammatory locally by intravitreal injection of zymasan which macrophage activator leads to enhance axonal regeneration after optical nerve injury.
* Boosting neuronal activity: activation/expression of gene programs related to axonal growth suck cAMP Pathway. Injection of Gabapentinoid increases axonal regeneration in adult sensory neurons.
* chondroitinase-ABC
* Neurotrophins
* Nerve growth factors such as BDNF: axonal regeneration and myelination.

# Brain Repair and Cell Replacement

**CNS injury**

* Damage to grey and white matter.
* Cascade if deleterious events that can affect both cell body and axonal function.
* Necrosis cell death.
* Damage to underlying tissue.
* Opening of BBB or BSCB.
* Demyelination, axonal degeneration, formation of glial scar.

**TBI**

* One of the causes of PD including dementia
* Temporary or permanently impairment of cognitive physiological and psychological social functions.
  + Neural stem cells: totipotent stem cells. NPCs:
  + Subventricular zone (SVZ)
  + Striatum
  + Dentate gyrus
* 3 active regions for neurogenesis (new neurons)

**Primal stem cells**

* multipotent or totipotent.
* can replace all types of cells: neurons, astrocytes, oligodendrocytes.
* Ability to target many neuroprotective or neurogenerative mechanisms.
* Can adapt to environment, evolve with pathology.
* Unlimited survival.
* After CNS injury: fetal tissue transplantation promotes recovery and combat neurodegenerative diseases. Animal receiving tissue has better outcome compared to cell suspension graft, improve survival.
* Tissue engineering approach for injection of suspension of stem cells.
* Hydrogel system to form a 3-D scaffold.

# Stem Cells Therapy

* BDNF, NGF, GDNF: transplant cell may secrete several trophic factors.
* **Embryonic stem cells**
  + Pluripotent
  + Almost produce any cell types
  + Can differentiate indefinitely
* **Multipotent stem cells**: hematopoietic stem cells => bone marrow, mesenchymal stem cells
* **Unipotent stem cells** - Neural stem cell found in 3 zones in the brain
* **MSCs** (mesenchymal stem cells)
  + Proliferate early
  + Generally. Located perivascular regions
  + Can differentiate into but not all cell types
* **iPSC**

Obtained from adult somatic cells reprogrammed to express factors of multipotency.

**Obstacles**

* Lack of migration of the transplanted stem cells.
* Low integration in. the host brain.
* Level of stem cell differentiation once transplanted.
* Difference in architecture during development (brain structured to help cells migrate) as opposed to the one in adulthood.
* Maturity of grafted cells at time of transplantation.
* In adult brain, cells capable of extending neurites and show axonal growth but weaker.
* Polysialic acid (PSA) enhances cell sensitivity to migration guidance cues.
* Decrease cell to cell interaction to promote plasticity and migration.
* Combination of different cells or therapeutic strategies in same graft could result in successful strategy.
* Glial cell precursors provide support and protection to neurons (myelin)
* Use of viral virus: express oncogenes.
* Intravenous is discouraged: cells can end up in undesired locations: heart, liver.
* Activation of immune system: cell therapy needs immune-suppressants.

# Experimental Approach to Cell Engineering and Reprogramming

* Small molecule inhibitors: SMA, NOTCH, FGF
* Differentiated for a week
* Day 8: transferred in mice
* **Allogeneic stem cell therapy**
  + Stem cells come from a donor NSCs or NPCs adult stem cells
  + All stem cells have the potential to induce an immune response in the host especially if route of administration done outside the nervous system
* **Autologous**
  + Stem cells belong to the subject, adult stem cells iPSCs.
  + Also stem cells are used to correct existing conditions.
* **GPCs**: Glial Progenitor cells can give rise to astrocytes and oligodendrocytes.
* **Transcription factors**

**MyOD**: induces differentiation into muscle cells.

**C/EBPalpha:** induces lymphocytes B to macrophages.

* Combination of TFs: ASC/1 +. BRN2 + Myt1L: transdifferentiate fibroblast into neurons.
* Direct reprogramming in vitro more efficient compared to in-vivo
* **Small molecules siRNA/miRNA**
  + Used to silence important some transcription factors.
  + Remove epigenetic DNA modification responsible of gene silencing.
  + Reactivate expression of particular factors.
* **In-vivo reprogramming**
  + Possible thru the use of cell fusion mechanism.
  + Bone marrow cells can fuse with neuronal cells in murine brain and stimulate reprogramming and push neurons to regenerate.
* Differences of efficiency between in-vitro and in-vivo maybe due to local microenvironmental conditions for ex. ECM presence.
* SASP (senescent associated secretory phenotype) reinforces senescent cells to promote tissue remodeling, senescent cells stimulate regeneration and cellular plasticity thru secretion of soluble factors.
* Nano-transfection DNA: based on focus of electrical field applied to skin: successful it generated fibroblasts into endothelial cells. Method seem less effective than the use of viruses.

# Miscellaneous

## How to knock out a gene in cells (transient silencing)

* Antisense oligonucleotide: a sequence complimentary to RNA/DNA of the gene of interest. They bind to the messenger and either block its translation or make the messenger a substrate for RNAseh which is an enzyme that can degrade this RNA.
* siRNA: small double-stranded RNA complementary sequence to targeted RNA, will bind to it and induce messenger degradation thru the enzyme Risc.
* Ribozyme: molecules of RNA, once bound to target induce cleavage. They are synthetized with the sequence complementary to the targeted RNA.
* The compounds are added to the cell media, cross the cellular membrane and bind to RNA messenger.
* Ribozymes are less stable and bigger and we use transfection liposomes.

## CRISPR/Cas9 technology

* A system of programmable RNA molecules that help tp guide an associated Cas9 in the nucleus to a specific target.
* 2 components
* SgRNA is an RNA sequence complementary to the target gene that needs to knock-out.
* sgRNA guides Cas9 in the nucleus to cleave both strands of DNA in a sequence specific manner.
* Following double-strand break, the genome is then repaired by the double-strand repair mechanism, for ex, error-prone non-homologous end joining mechanism (NHEJ).

## iPSc

* Models for study disease.
* Model for normal developments.
* Screening for mutagens.
* Understanding tissue repair
* Understanding regeneration
* Protocols for cell therapy for human diseases
* Ideals since cells can be derived from patient and avoid rejection from immune system and post-transplant immune-suppressant drugs: cells will migrate to the affected area and regenerate tissues.