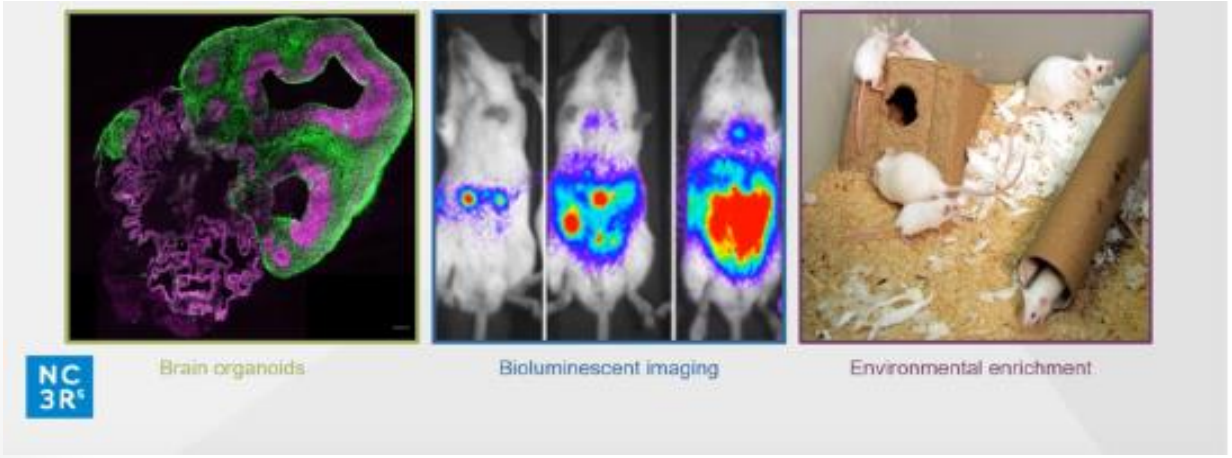
THE PRINCIPLES OF HUMANE EXPERIMENTAL TECHNIQUE

The principles of the 3Rs:

- REPLACEMENT
- REDUCTION
- REFINEMENT

Principles of the 3Rs



The Principles of Humane Experimental Technique
W.M.S. Russell and R.L. Burch , 1959

	Standard	Contemporary
Replacement	Methods which avoid or replace the use of animals	Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals
Reduction	Methods which minimise the number of animals used per experiment	Appropriately designed and analysed animal experiments that are robust and reproducible, and truly add to the knowledge base
Refinement	Methods which minimise animal suffering and improve welfare	Advancing research into animal welfare by exploiting the latest *in vivo* technologies and by improving understanding of the impact of welfare on scientific outcomes

3Rs impacts are wide ranging, from policy and regulatory change to the development and uptake of new technologies and approaches.

Alternatives to animal testing are important for:

- Ethical issues
- Costs
- Standard procedures

EURL ECVAM

European Union Reference Laboratory for alternatives to animal testing

- EURL ECVAM is actively involved in the development and promotion of alternative approaches to animal testing with the aim to replace, reduce or refine the use of laboratory animals in the safety assessment of chemicals and the quality control of biologicals (e.g. vaccines).

- EURL ECVAM is an integral part of the Joint Research Centre (JRC), the science and knowledge service of the European Commission and is located at the JRC site in Ispra, Italy.

- The current activities of EURL ECVAM build on over 25 years of JRC include:
  - conducting research and collaborating in EU and international research initiatives;
  - coordination and undertaking of validation studies of alternative methods for the safety assessment of chemicals;
  - dissemination of information and sharing of knowledge across disciplines and sectors;
  - promotion of alternative methods and the Three Rs in an international context.

Eye irritation tests

In vitro

- Eye irritation:
  - HET-CAM (hen's egg chorio-allantoic membrane) test

  - BCOP (bovine corneal opacity) test
  - ICE (isolated chicken eye) test

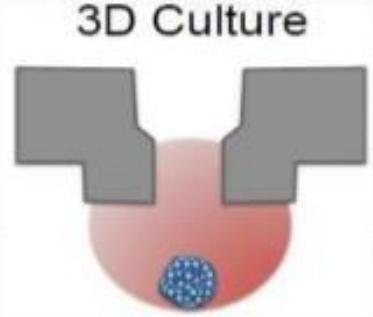
In vivo

- Draize test in rabbit's eye

Alternative models
(cell culture, stem cell, c. *elegans*, zebrafish)

Applications in Pharmacology

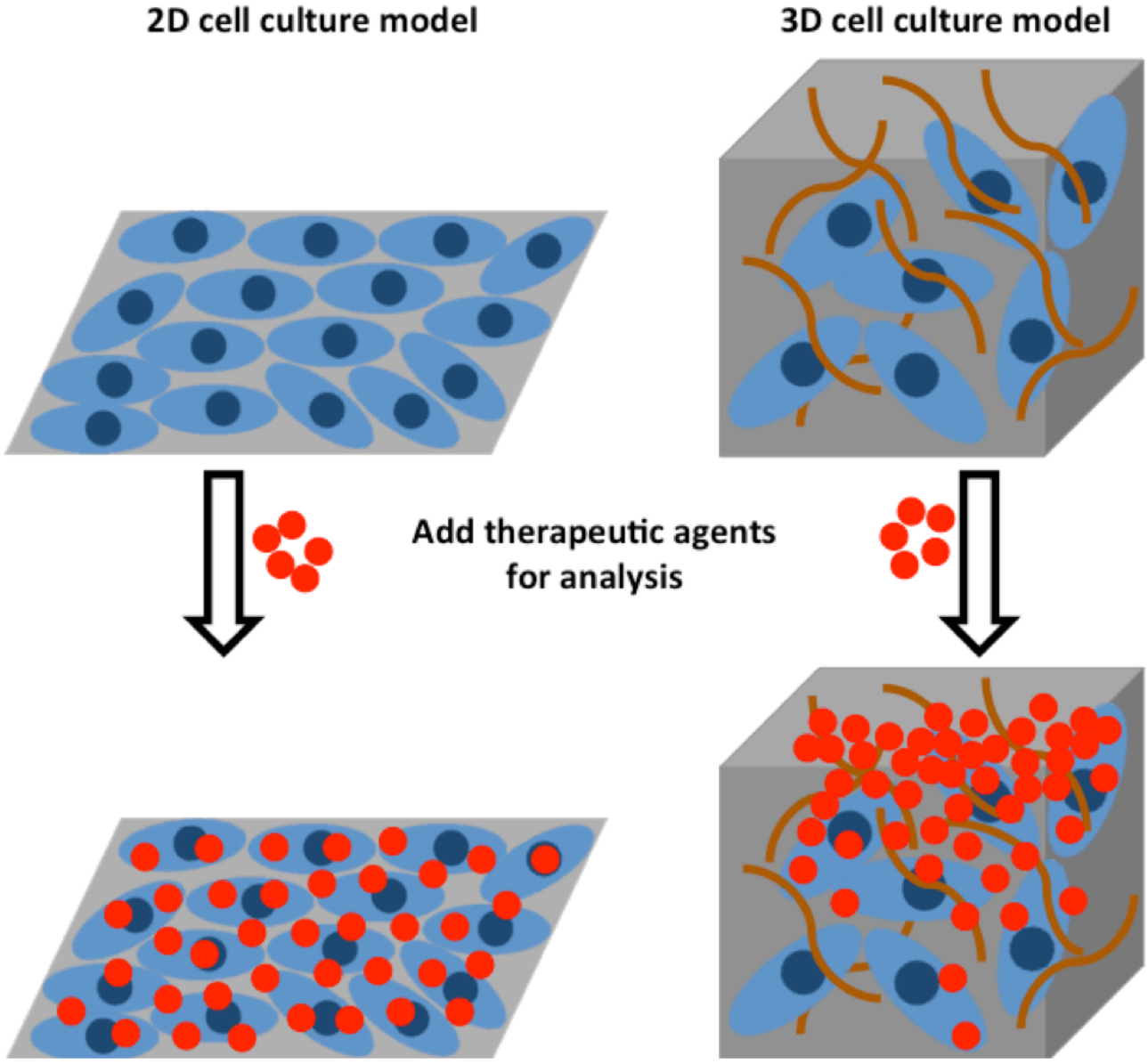
3D vs. 2D cell culture



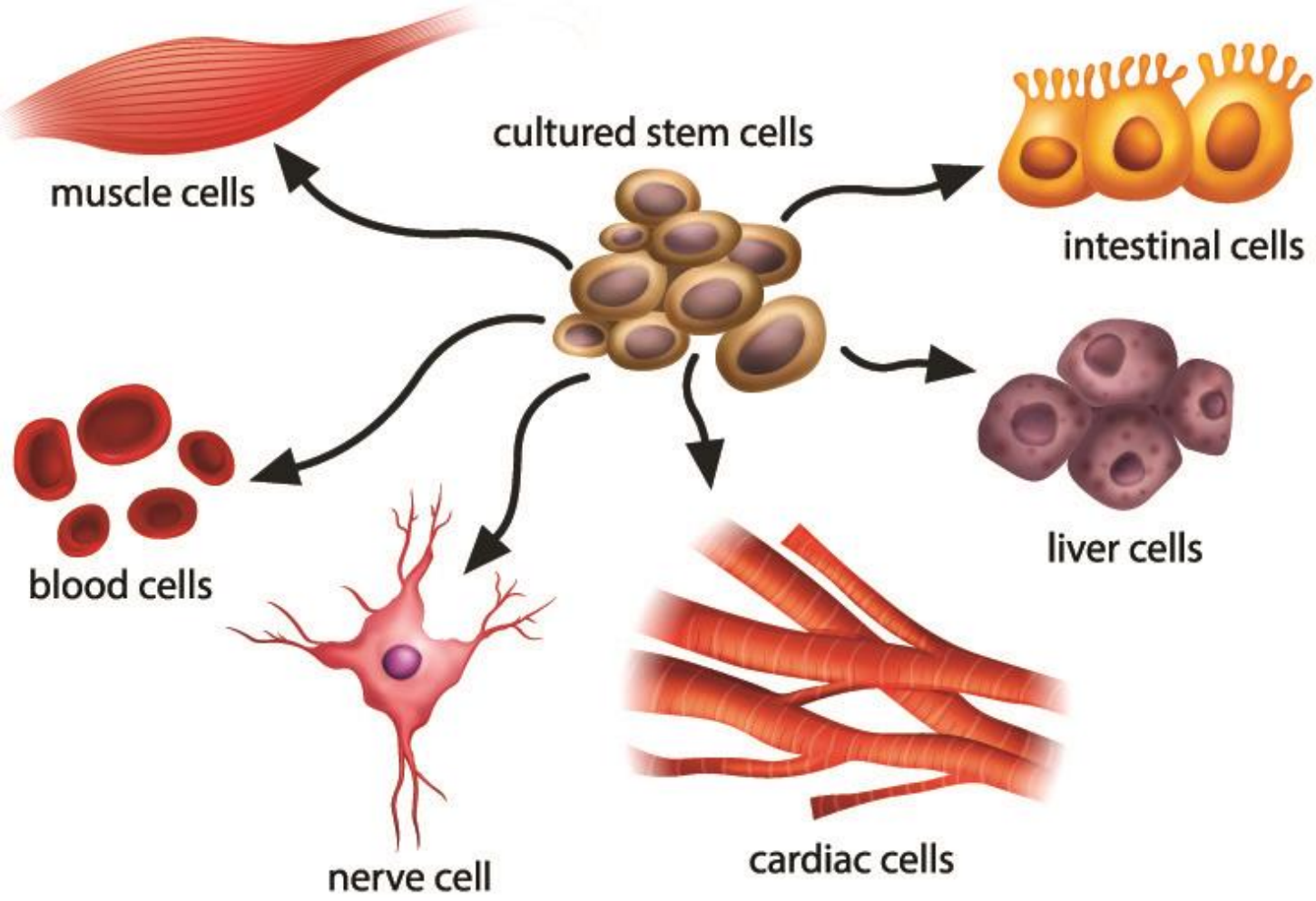
VS

- Physiologic cell-to-cell contact dominates
- Cells interact with extracellular matrix (ECM)
- Diffusion gradient of drugs, oxygen, nutrients, and waste
- Co-culture of multiple cell mimics microenvironment
- Shows resistance to anticancer drug as in vivo tumor

- Cell-to-cell contact only on edges
- Cell mostly in contact with plastic
- Cells contact extracellular matrix mostly on one surface
- No gradients present
- Co-culture unable to establish a microenvironment
- Anticancer drug resistance is not seen


2D cell culture model
3D cell culture model
Add therapeutic agents
for analysis

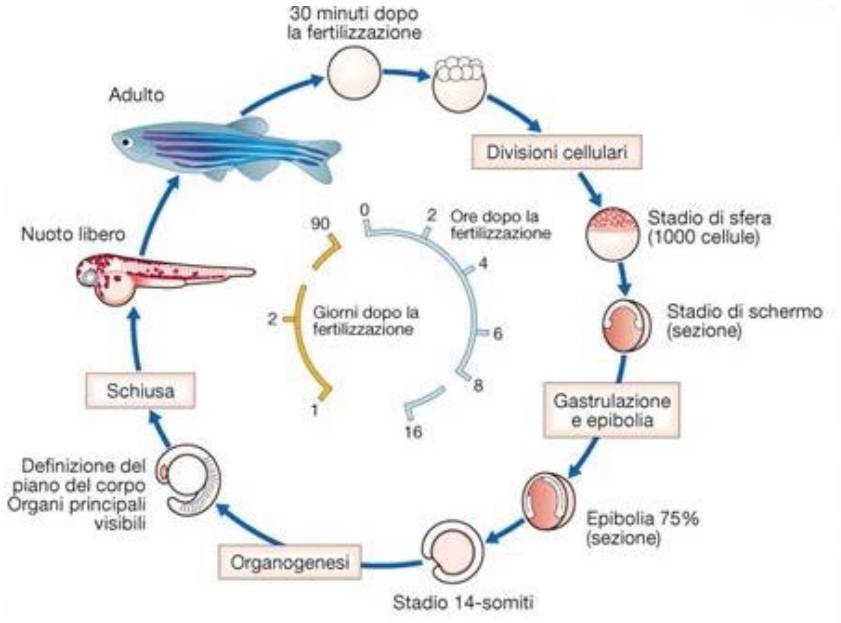
Human Stem Cell Applications



Zebrafish

Adult fish, which is about 2.5 cm long

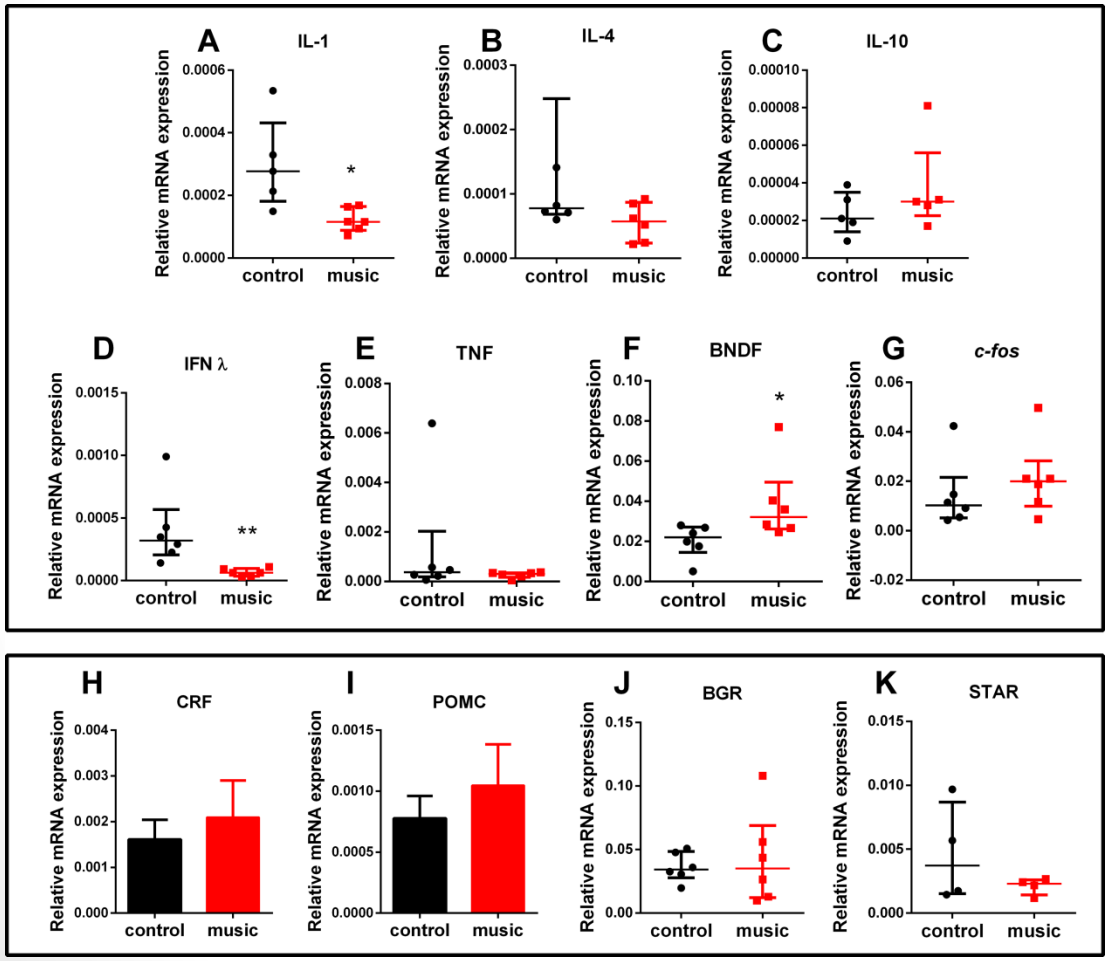
- Freshwater fish native to South Asia
- Important and widely used vertebrate model organism in scientific research
- It has been modified by researchers to produce many transgenic strains



Uova fecondate a diversi stadi dello sviluppo

The effects of auditory enrichment on zebrafish behavior and physiology

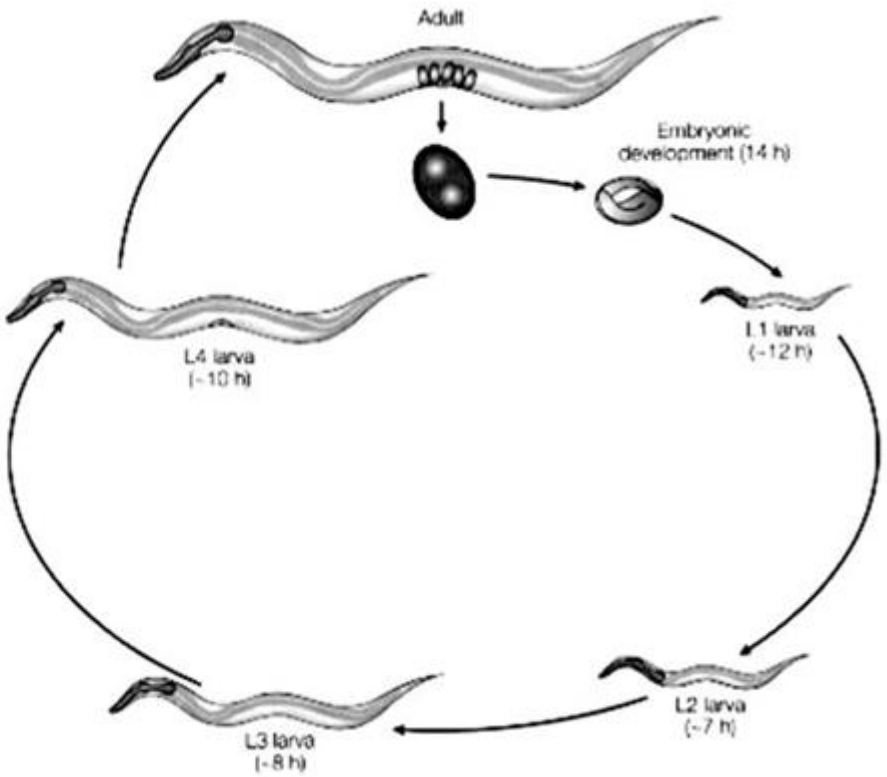
Heloísa H. A. Barcellos[1,2,*], Gessi Koakoski[3,*], Fabiele Chaulet[4,*], Karina S. Kirsten[3,*], Luiz C. Kreutz[2,3,*], Allan V. Kalueff[5,6,7,8,9,10,11,12,13,*] and Leonardo J. G. Barcellos[1,2,3,4,6]



Barcellos et al., PeerJ. 2018 Jul 23;6:e5162.

Nematode Caenorhabditis Elegans (C. Elegans)

Sydney Brenner, born 1927, La Jolla, CA, USA.



Caenorhabditis elegans:

- It is a free-living, transparent nematode, about 1 mm in length, that lives in temperate soil environments
- It offers an attractive genetic system to study various aspects of development, including the developmental regulation of cell proliferation
- It was the first multicellular organism to have its whole genome sequenced

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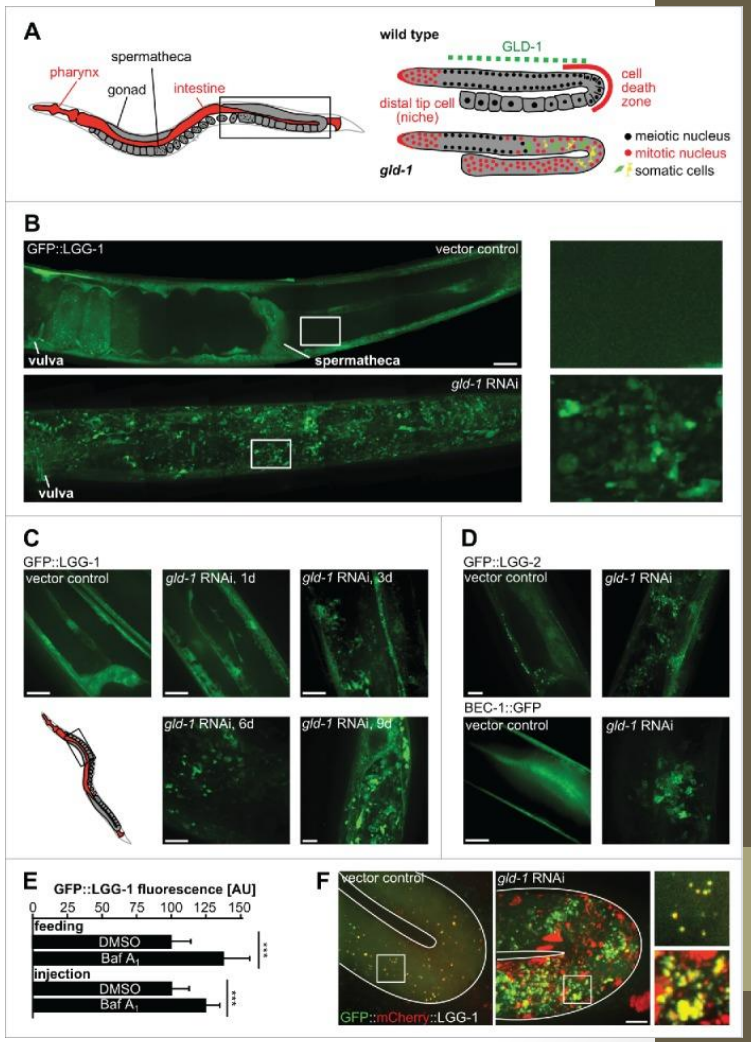
BASIC RESEARCH PAPER

OPEN ACCESS

Autophagy and modular restructuring of metabolism control germline tumor differentiation and proliferation in *C. elegans*

Ligia C. Gomes[a,b,#], Devang Odedra[a,b], Ivan Dikic[a,b,c], and Christian Pohl[a,b]



Published in final edited form as:
Neurochem Int. 2016 September ; 98: 122–128. doi:10.1016/j.neuint.2016.01.008.

Acute Blockade of the *C. elegans* Dopamine Transporter DAT-1 by the Mammalian Norepinephrine Transporter Inhibitor Nisoxetine Reveals the Influence of Genetic Modifications of Dopamine Signaling *In Vivo*

Daniel P. Bermingham[1], J. Andrew Hardaway[1], Chelsea L. Snarrenberg[1], Sarah B. Robinson[1], Oakleigh M. Folkes[1], Greg J. Salimando[1], Hussain Jinnah[1], and Randy D. Blakely[1,2]

When *replacement is not possible*, *reduction* e *refinement* are essential

Animals Research Guidelines

ARRIVE
(Animal Research: Reporting of In Vivo Experiments)

PREPARE
(Planning Research and Experimental Procedures on Animals: Recommendations for Excellence)

ARRIVE

The ARRIVE guidelines , originally published in PLOS Biology, were developed in consultation with the scientific community as part of an NC3Rs initiative to improve the standard of reporting of research using animals.

https://www.nc3rs.org.uk/arrive-guidelines

PREPARE

Norecopa is a member of ecopa (European Consensus-Platform for Alternatives).

Norecopa has produced the PREPARE guidelines for planning animal research and testing and supports National Consensus Platforms for the 3Rs.

https://norecopa.no/about-norecopa

Animal models of human diseases

Animal models of neurodegenerative diseases

Animal models of adult-onset neurodegenerative diseases have enhanced the understanding of the molecular pathogenesis of some of the main neurodegenerative disorders that inexorably progress to severe disability and death:

- Alzheimer's disease (AD)
- Parkinson's disease (PD)
- Frontotemporal dementia(FTD)
- Amyotrophic lateral sclerosis (ALS)

Though in many individuals these neurodegenerative disorders have no clear genetic causes, the field has been guided by the discovery of mutated genes that deterministically drive these disorders, as well as genetic variants that alter risk.

No animal model of AD, PD, FTD, or ALS fully phenocopies human disease. Many models recapitulate the initial proteinopathy or other pathological features linked to the human disorder.

Alzheimer's disease (AD)

- AD is typically characterized by early progressive memory loss followed by impairments in executive functions and other behavioral disturbances and other cognitive abilities serious enough to interfere with daily life. AD accounts for 60-80 % of dementia cases.

- AD is characterized by three hallmark pathologies:
  1. senile plaques (whose major insoluble component are fibrillar aggregates of Aβ)
  2. neurofibrillary tangles
  3. hippocampal and cortical neurodegeneration

- Mutations in the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) are the main causes of autosomal dominant early-onset AD, while the APOEε4 allele is a major risk factor for late-onset AD.

Alzheimer's disease (AD)

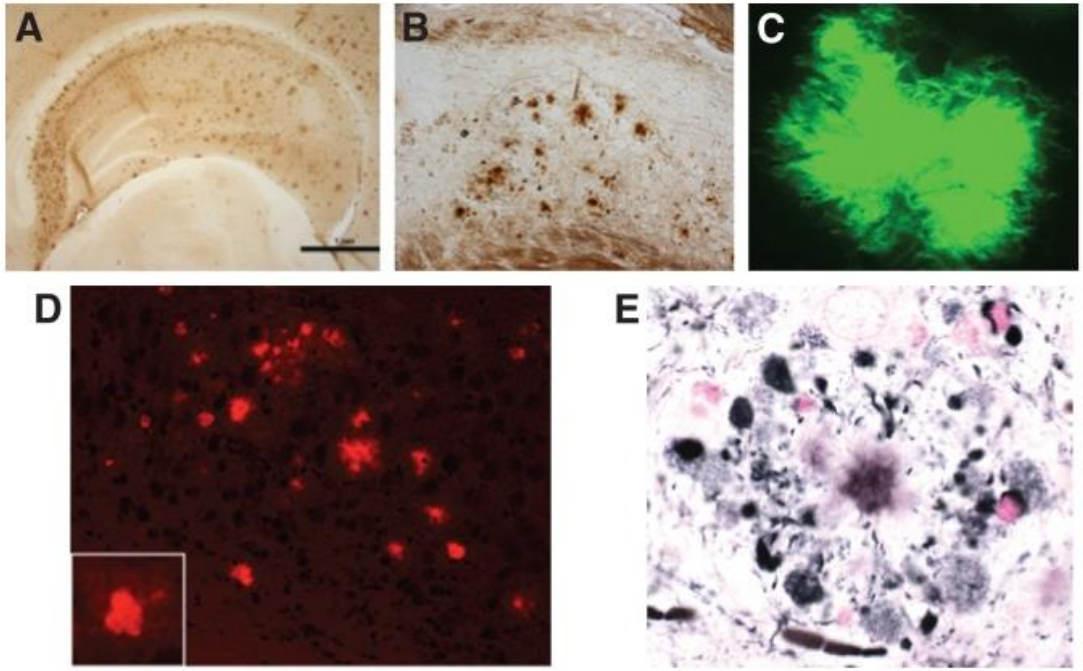
Genetic-Based Models of Amyloid Pathology

Transgenic rodents that drive Aβ aggregate accumulation, model amyloid deposition in senile plaques and in some cases cerebrovascular amyloid.

AD-linked human mutations in APP, PSEN1 and PSEN2, function in the mouse model much as they do in human.

- APP mutants either increase total Aβ or more commonly
- PSEN½ mutants alter endogenous processing of mouse APP, but do not lead to amyloid deposition. Co-expression of PSEN½ mutants with an APP transgene dramatically accelerates the amyloid phenotype by increasing the relative production of Aβ42.

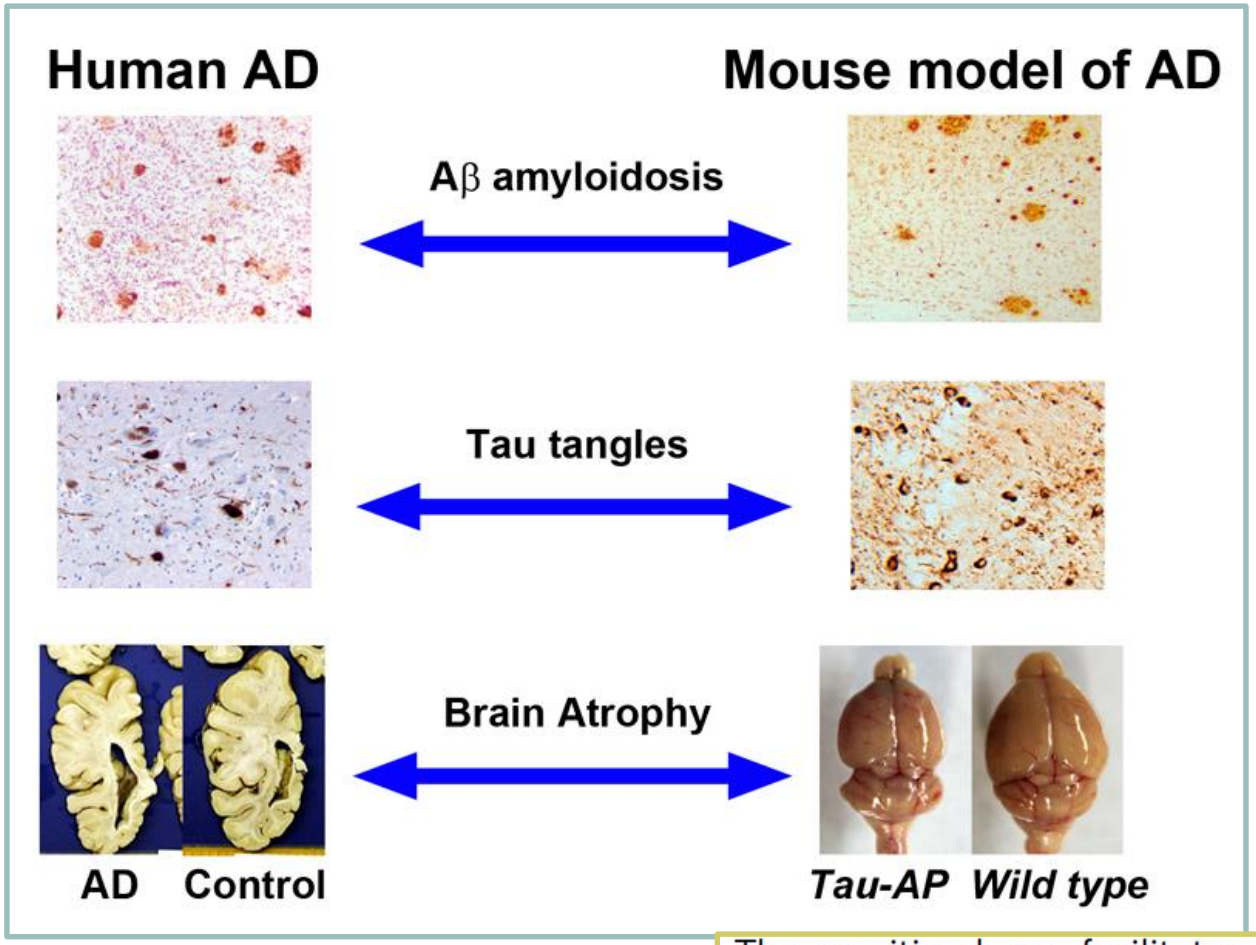
Aβ plaques found in the brains of AD transgenic mice are structurally similar to those found in the human brain; they initiate as diffuse plaques consisting mainly of Aβ42, develop a dense Aβ42 core, and then incorporate Aβ40, as well as numerous other non-Aβ components such as ubiquitin and α-synuclein. As in the human brain, these plaques stain positive with both thioflavin and Congo red, and show similar fibrillar structures by microscopy.



Frank M. LaFerla and Kim N. Green. *Animal Models of Alzheimer Disease.* Cold Spring Harb Perspect Med. (2012)

Visualization of amyloid plaques in 3xTg-AD mice with classical stains. 3xTg-AD mice develop diffuse and fibrillar plaques, as detected with antibody 6E10 (A and B), thioflavin-S (C), Congo red (D), and Gallyas stain (E).

Li and his colleagues showed how amyloid plaques influence tau tangles, two key features in brains of Alzheimer's, to provoke death of nerve cells and brain atrophy that underlies memory loss. This new model will help scientists to better understand the root cause of this devastating illness and for preclinical drug screening.



The neuritic plaque facilitates pathological conversion of tau in an Alzheimer's disease mouse model

Tong Li[1], Kerstin E. Braunstein[1], Juhong Zhang[1], Ashley Lau[1], Leslie Sibener[1], Christopher Deeble[1] & Philip C. Wong[1,2]

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Acute and chronic cannabinoid extracts administration affects motor function in a CREAE model of multiple sclerosis

Elena Buccellato[a], Donatella Carretta[a], Aneli Utan[a], Chiara Cavina[a], Ester Speroni[a,*], Giampaolo Grassi[b], Sanzio Candeletti[a], Patrizia Romualdi[a]

[a] Department of Pharmacology,
[b] CRA-CIN, Council in Agricultur

ABSTRACT

Aim of the study: The multiple sclerosis is an immuno-mediated disorder of the Central Nervous System characterized by inflammatory processes and neurodegenerative changes. It has been shown that the endocannabinoid system is altered in this disease and that the exogenous cannabinoids may play a possible role in its therapeutic management. The aim of the present study was to investigate the efficacy of crude extracts of *Cannabis sativa* on motor symptoms in the chronic relapsing experimental autoimmune encephalomyelitis (CREAE), a murine model of multiple sclerosis.

Materials and methods: CREAE-induced mice were injected by different crude ethanolic extracts from *Cannabis sativa*, containing Δ^9-tetrahydrocannabinol, cannabidiol, or cannabinoid-free, respectively. The effect of the combined treatment with Δ^9-tetrahydrocannabinol and cannabidiol extracts has also been investigated. All extracts were administered in acute and chronic experimental protocols.

Results: The chronic administration of Δ^9-tetrahydrocannabinol-rich extract resulted in a significant reduction of neurological deficits that lasted until the end of the observations. The acute and chronic treatments with the cannabidiol-rich extract resulted unable to induce changes of neurological signs. However, during the relapse phase a significant decrease of neurological scores was observed. The combined treatment with Δ^9-tetrahydrocannabinol and cannabidiol extracts was ineffective, whereas the acute administration of the cannabinoid-free extract showed a significant improvement.

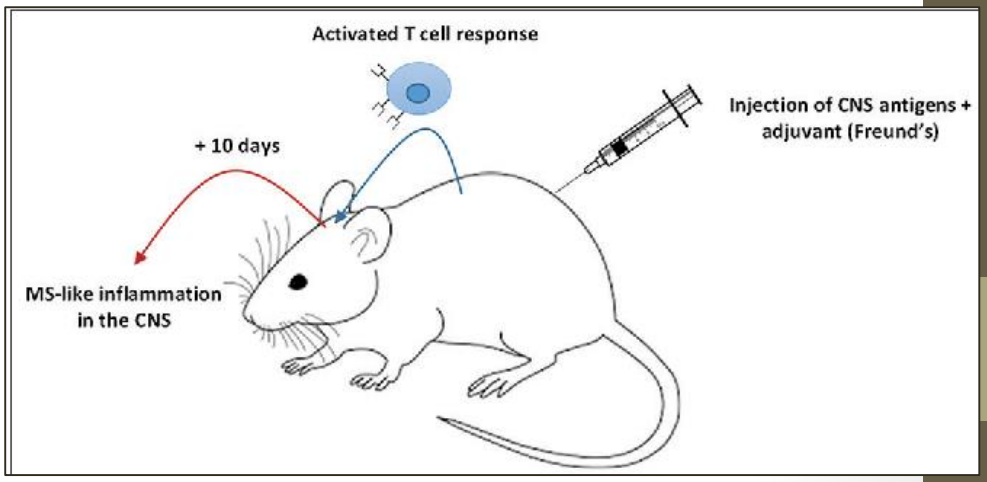
Conclusions: Our study shows a patchy effect of different cannabinoid extracts on CREAE-induced motor deficits. Although the effect of crude extracts of *Cannabis sativa* here reported need to be further investigated to define the exact therapeutic target of each cannabinoid, it may represent a possible therapeutic approach for the management of the multiple sclerosis.

MULTIPLE SCLEROSIS: experimental allergic encephalomyelitis (EAE)

Chronic/relapsing experimental allergic encephalomyelitis (CREAE) was induced in Biozzi AB/H animals according to the Baker protocol (**Baker et al., 1990**).

Experimental allergic encephalomyelitis (EAE) was induced in Biozzi AB/H (antibody high) mice by sensitization with spinal cord homogenate in Freund's incomplete adjuvant, containing a mixture of *Mycobacterium tuberculosis* H37Ra and *Mycobacterium butyricum.* Injections were performed on day 0 and day 7.

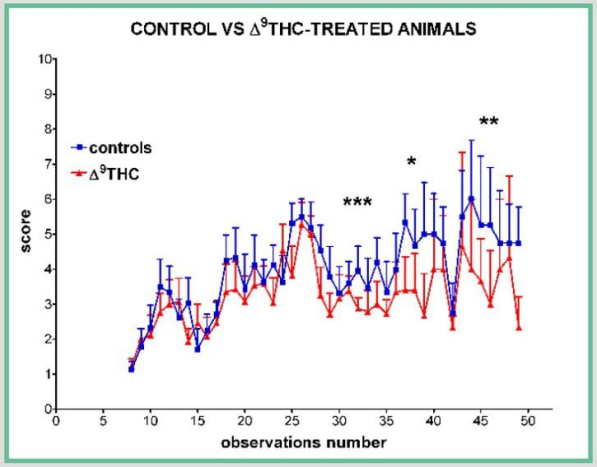
Biozzi AB/H mice were highly susceptible to EAE induction and followed a chronic relapsing pattern of disease. Disease episodes were characterized by **mononuclear infiltration** of the central nervous system, with **demyelination** being particularly evident in relapse. The cellular infiltrates, which were associated with immunoglobulin deposition, consisted of **macrophages** and **CD4 T-lymphocytes.**

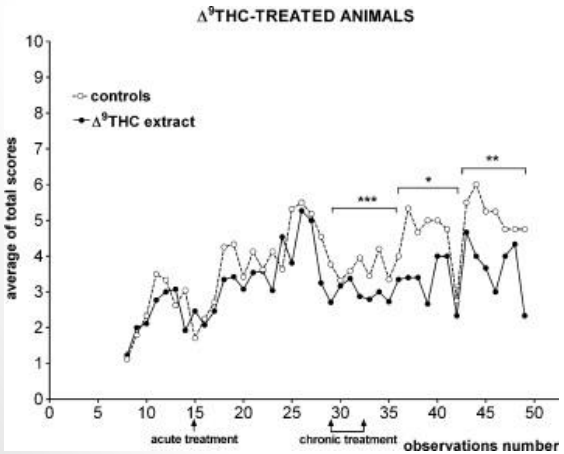


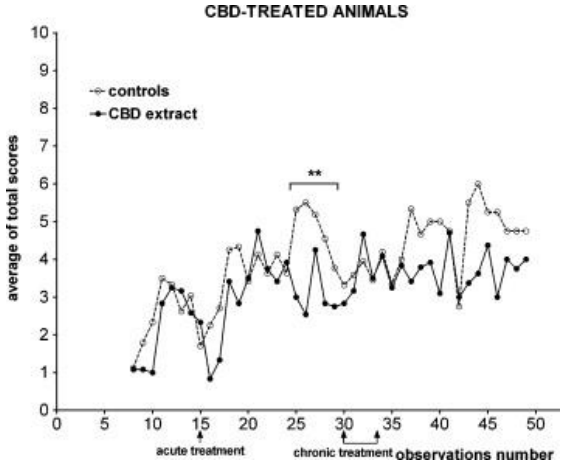
Cannabinoid extracts on motor function in a model of multiple sclerosis. We reported the efficacy of Δ^9-tetrahydrocannabinol (Δ^9THC), cannabidiol (CBD), and flavonoid extracts from *Cannabis sativa* on different phases of the experimental disease.

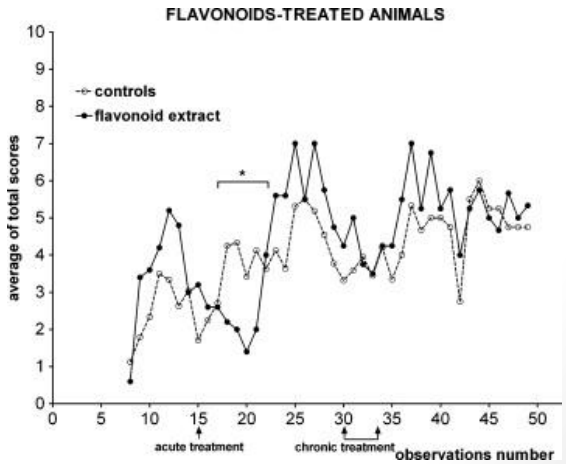
acute phase --- remission --- relapse1 --- remission

acute treatment chronic treatment









Strategies for the development of new animal models for neurodegenerative disorders

- Efforts to better model various non-genetic factors and co-morbid diseases that commonly occur in humans suffering from various neurodegenerative disorders might be warranted. We should recognize that the relatively **sterile environments**, in terms of behavioral enrichment, microbiome, and environmental exposures, **do not represent the exposure history of humans who develop neurodegenerative conditions**.

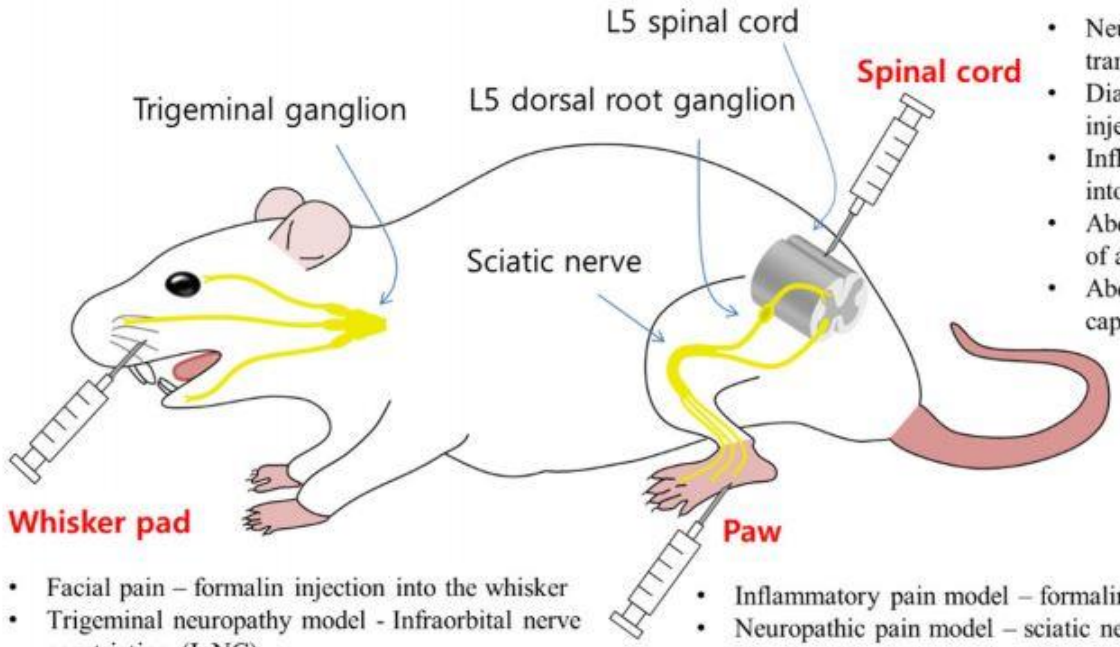
- More studies should be conducted in the setting of old models that better **mimic the physiology of aging typically present in humans with these diseases**. Given the expense associated with such studies, like the aging rat colonies, the field may consider a standard resource that generates such aged models to enable more standard use.

- We should make a more concerted effort to understand and model selective **CNS-cell vulnerability**. Though often thought of in terms of neuronal vulnerability, a major gap in knowledge relevant to these human disorders is that despite widespread expression of a given mutant protein, not all areas of the brain degenerate at equivalent rates. Such selective regional or cellular vulnerability is often not carefully considered in most of our current models.

- Use of novel tools and techniques (e.g., gene editing) to **generate models in mammals other than mice** should be considered, but only when an appropriate underlying hypothesis frames the study. Development of such models will inherently be expensive.

Dawson et al., *Nat Neurosci.* 2018

PAIN



- Neuropathic pain model – sciatic nerve transection (CCI)
- Diabetic neuropathy pain model - intraperitoneal injection of streptozotocin
- Inflammatory pain model – formalin injection into paw
- Abdominal pain model – intraperitoneal injection of acetic acid
- Abdominal pain model - Intracolonic injection of capsaicin

- Facial pain – formalin injection into the whisker
- Trigeminal neuropathy model - Infraorbital nerve constriction (IoNC)

- Inflammatory pain model – formalin injection into paw
- Neuropathic pain model – sciatic nerve transection (CCI)
- Neuropathic pain model – L5 ventral root transection (VRT)
- Hyperalgesia model – Carrageenan injection into paw
- Peripheral neuropathic pain model - intraperitoneal injection of paclitaxel
- Diabetic neuropathy pain model - intraperitoneal injection of streptozotocin
- Mirror pain model - acidic saline injections into the right gastrocnemius muscle

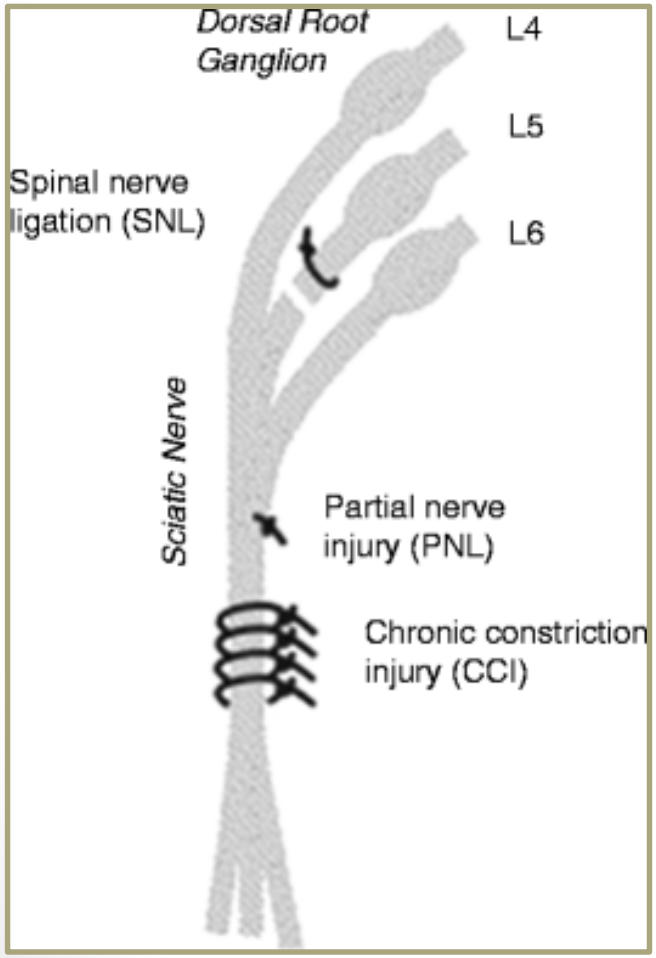
NEUROPATHIC PAIN MODELS

Table 1 Neuropathic pain models[2]

Model name	Type of injury	Animal
Axotomy	Complete sciatic section	Rat
Chronic sciatic constriction	4 ligatures around the nerve	Rats and mice
Partial sciatic ligature	Ligature of 1/3 to ½ the nerve	Rats and mice
Spinal roots ligation	1. Ligation of L5/L6	Rats
	2. Ligation of L7	Monkeys
Nerve-sparing injury	Tibial and peroneal axotomy	Rats and mice
Tibial and sural nerve transection	Tibial and sural axotomy	Rats
Common peroneal ligation	Common peroneal ligation	Mice
Sciatic cryoneurolysis	Nerve freezing	Rats
Caudal trunk resection	Caudal trunk resection	Rats and mice
Sciatic inflammatory neuritis	Zymozan injection, TNF around the nerve	Rats and mice
Balloon-induced sciatic injury	Implant of polyethylene balloon around the nerve	Rats and mice
Laser-induced sciatic injury	Decreased blood flow to the nerve mediated by radiation	Rats
Spinal injury by contusion	A weight is dropped on exposed spinal cord	Rats and mice
Excitotoxic spinal cord injury	Spinal injection of aminoacids	Rats and mice
Spinal hemisection	Laminectomy of T11-T12	Rats
Drug-induced	Direct drug injury to peripheral nerves	1.Rats, mice, Guinea pigs
1. antineoplastic drugs (vincristine, cisplatin,		
oxaliplatin, paclitaxel)		
2. anti-HIV (2,3- dideoxycytidine, didanosine)		2. Rabbits, rats
Diabetes-induced neuropathy	Persistent changes in nerves induced by hyperglycemia	Rats and mice
1. induced by streptozocin		
2. genetic models		
Bone pain models	Inoculation of cancer cells in bones	Rats and mice
HIV-induced neuropathy	Inoculation of HIV gp120 protein in sciatic nerve	Rats
Postherpetic neuralgia	Injection of viral cells	Rats
Alcoholic neuropathy	Ethanol administration for long periods	Rats
Pyridoxine-induced neuropathy	Administration of high doses of pyridoxine for long periods	Dogs and rats
Trigeminal neuralgia	Trigeminal compression; chronic infraorbitary nerve constriction	Rats
Orofacial pain	Formalin of carrageenin injection in temporomandibular joint and jaw	Rats and mice
Acrylamide-induced	Administration of acrylamide for long periods	Rats

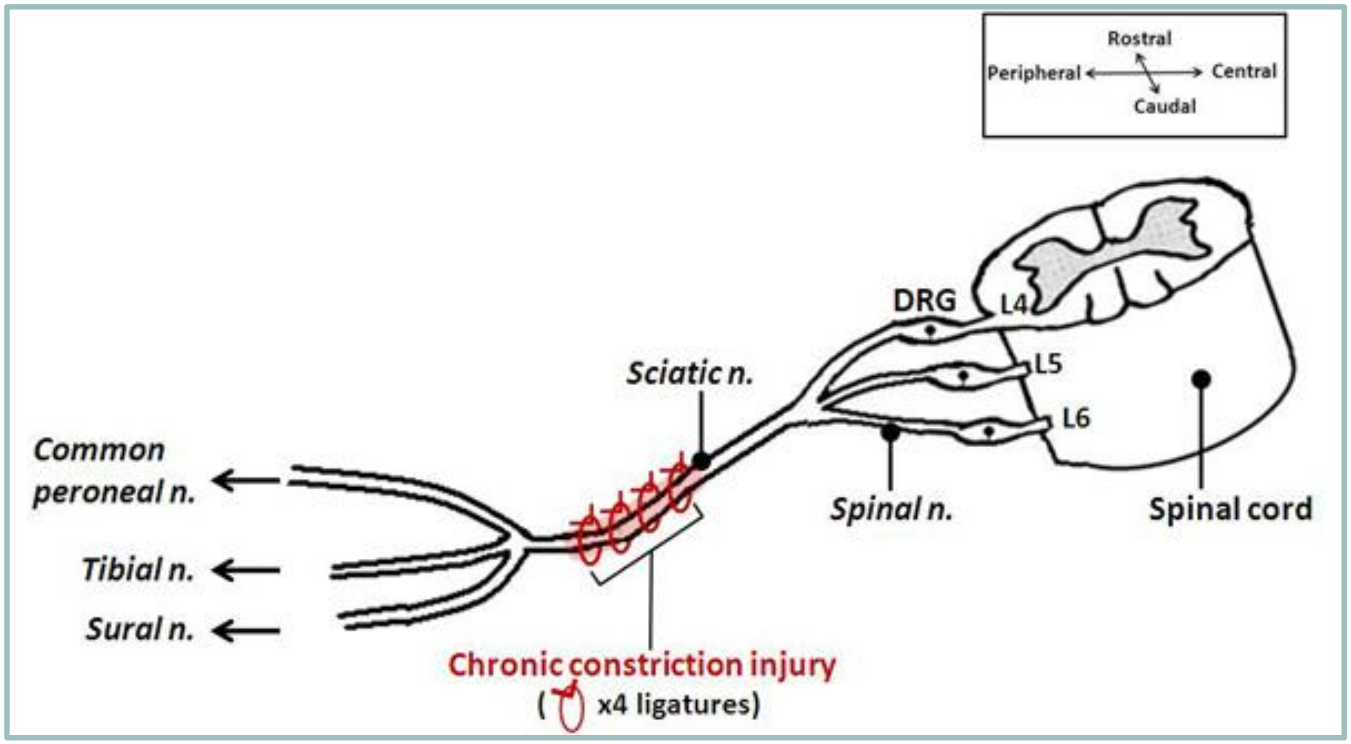
Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. Fundam Clin Pharmacol. 2011;25(1):1-28

Neuropathic Pain



- **PNL: Partial sciatic nerve ligation**
  (Seltzer, Dubner and Shir, 1990)
- **SNL: Spinal nerve ligation**
  (Kim and Chung, 1992)
- **CCI: Chronic Constriction Injury**
  (Bennett and Xie, 1988)

CHRONIC CONSTRICTION INJURY (CCI)



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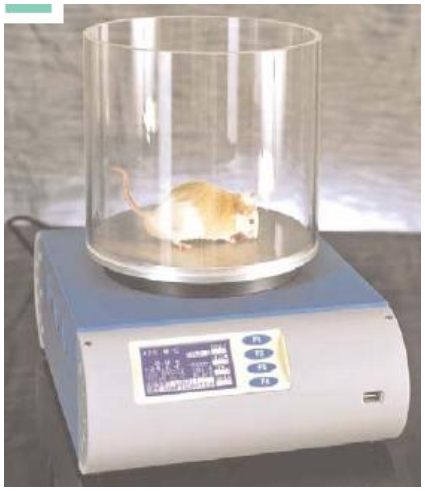
METHODS USED TO EVALUATE PAIN BEHAVIORS IN RODENTS

VON FREY TEST



HOT/COLD PLATE

TAIL FLICK

PLANTAR TEST