BEHAVIOURAL PARADIGMS IN MICE FOR THE STUDY OF AUTISM-RELATED DISORDERS

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Some of the associated symptoms of autism

- Theory of Mind impaired
- Mental retardation
- Language delays
- Anxiety
- Motor clumsiness
- Idiosyncratic hypersensitivity to sensory stimuli
- Seizures
- Larger brain volume in early development
- Fewer Purkinje cells in the cerebellum
- Reduced activation of the fusiform gyrus and amygdala while engaged in social tasks during fMRI imaging
Autism is a neurodevelopmental disorder that is behaviourally defined by three core symptoms:

° Deficits in reciprocal social interaction
° Qualitative impairments in social communication
° Stereotypies, repetitive, ritualistic behaviors, restricted interests
Autism is a neurodevelopmental disorder that is behaviourally defined by three core symptoms:

- deficits in appropriate social interaction
- impaired communication
- aberrant repetitive behavior

Mouse behavioural tasks that may model additional features of autism include tests relevant to:
- anxiety,
- sleep disturbances (circadian cycle),
- elevated pain threshold,
- self-control and impulsivity,
- stereotypies, repetitive, ritualistic behaviors, restricted interests
Mutations in the X-linked Methyl-CpG-Binding Protein 2 (MeCP2) gene account for Rett syndrome, a severe neurodevelopmental disorder and a genetic cause of mental retardation.

RTT is associated with a wide variety of specific symptoms. These include:

- breathing irregularities;
- episodes of seizures;
- gait dyspraxia;
- back deformities;
- stereotyped hand movements;
- sleep problems;
- autistic-like behaviours.

Different developmental phases can be identified in Rett patients.
Subtle deficits in young infants include: hypotonia, poor suck, weak cry, abnormal general movements.

Studies of family home videos, recorded before the disorder was clearly manifested, would confirm that girls with RTT, during the first months of life, are not so asymptomatic as they were thought.
If behavioural characterization starts at adulthood, we could miss some important information:

**The earlier the better:**

**Phenotyping the presymptomatic phase**

- During the first postnatal weeks important brain regions and behavioral competencies are still far from their adult shape;
- Only during the first postnatal weeks it is possible to evaluate variation in temporal patterns (delays or accelerations in the onset and maturation of sensory, motor and cognitive responses).

_Branchi, Bichler, Berger-Sweeney, and Ricceri 2003_
Ontogenic profile of ultrasonic vocalization production

MeCP2-308 male pups emitted a significantly lower number of ultrasound vocalizations than wt controls.
On pnd 3, wt mice spent approximately the same amount of time in this activity from the first to the last minute of observation. By contrast, hz mice increasingly engaged in this activity as time passed (p = .048).

On pnd 6, wt mice showed less tremors as time passed. On the other hand, MeCP2-308 mice increased their engagement in this activity from the first to the last minute of observation (p = .006).
Behavioural development shown by MeCP2-308 mice appears delayed in the early phases of postnatal life.

1. **Locomotion**: general translocation of the body of at least 1 cm in the glass container;

2. **Pivoting**: locomotor activity involving the front legs alone and resulting in laterally directed movements;

3. **Wall climbing**: alternating forelimb placing movements on the wall of the container;

4. **Side**: no visible movement of the animal when lying on the back or the side;

5. **Head raising**: raising of the head up and forward;

6. **Nose probing**: the snout against floor or walls of the container;

7. **Floor paddling**: regularly alternating forelimb placing movements on the floor of the container, without locomotor movement;

8. **Curling**: while on side or back, vigorous side-to-side rolling movements, associated with a convex arching of back (head in a closer apposition to hump/hindlimb region);

9. **Immobility**: no visible movement of the animal when placed with all the four paws on the floor;

10. **Other**.
Mutant mice display an increased anxiety-like profile as measured in the light-dark test.

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Locomotor coordination deficits, delayed acquisition of spontaneous movements and impaired communicative behaviour have been observed in MeCP2-308 mutant mice already soon after birth:

The presence of behavioural alterations during the early phases of development suggest that also the “so-called” pre-symptomatic phase is worth of being investigated.

These alterations also represent precocious biomarkers to be exploited for early testing of new potential therapies.
Causes of Autism Spectrum Disorders: strongest evidence is genetic

- 4:1 frequency ratio boys:girls
- >60% concordance for monozygotic twins
- Linkage analyses indicate multiple genes underlying this complex disease, including candidate genes at loci on chromosomes 6, 7, 13, 15, 17
- Proposed candidate genes include engrailed-2, Neuroligin, FOXP2, WNT2, HOXA1, Fragile X, serotonin transporter polymorphisms, GABA receptor subunit GABRB3, Reelin and other developmental genes and transcription factors
Genome screens for loci that predispose to autism spectrum disorders.

° The figure illustrates the overlap of linkage signals from published genome screens. Weakly positive scores are included.
° The most consistent signal is on chromosome 7q (long arm), but there is also considerable overlap on chromosomes 2q and 16p (short arm).  (from: Folstein & Rosen-Sheidley, 2001)
Role of reduced Reelin function in autism

• Reelin is a large glycoprotein that is secreted into the extracellular matrix, is critical for laminar organisation of several brain structures during development (D'Arcangelo G, et al 1995).

• In about 45% of autistic patients a reduction of Reelin levels was observed in cerebellum and blood.

• It has been shown that individuals inheriting RELN alleles that contain more than 11 GGC repeats in the 5' UTR of the Reelin mRNA have an increased risk of autism (Persico AM et al 2001).
Table 2. Distribution and characteristics of microscopic neuroanatomical alterations in brains of autistic patients and reeler mice

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Autistic patients</th>
<th>Refs</th>
<th>Reeler mice</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>Increased cell density</td>
<td>[117]</td>
<td>Inversion of cortical lamination</td>
<td>[121,122]</td>
</tr>
<tr>
<td></td>
<td>Smaller cortical minicolumns</td>
<td>[118]</td>
<td>Neuronal disorganization</td>
<td>[121–125]</td>
</tr>
<tr>
<td></td>
<td>Ectopic neurons</td>
<td>[117]</td>
<td>Altered intracortical course of afferent fibers, with quantitatively normal thalamocortical connections</td>
<td>[124,125]</td>
</tr>
<tr>
<td></td>
<td>Neuronal disorganization</td>
<td>[117]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Areas of increased cortical thickness</td>
<td>[117]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor lamination in the anterior cingulate cortex</td>
<td>[119]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>Decreased Purkinje cell number</td>
<td>[117,119]</td>
<td>Decreased Purkinje cell number</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>Modest decrease in granule cell counts</td>
<td>[119]</td>
<td>Purkinje cells are disorganized</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Ectopic subcortical Purkinje cells</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Climbing fibers innervate more than one Purkinje cell</td>
<td></td>
</tr>
<tr>
<td>Deep cerebellar nuclei</td>
<td>Increased cell size before age 12 and decreased cell counts after age 22</td>
<td>[119]</td>
<td>Decreased cell counts and dysplasia in lateral nucleus (dentate nucleus in humans)</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>Dysplasia in the dentate nucleus</td>
<td>[117]</td>
<td>Subcortical ectopic gray matter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subcortical ectopic gray matter</td>
<td>[117]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inferior olivary nucleus</td>
<td>Increased cell size before age 12 and decreased cell size after age 22</td>
<td>[119]</td>
<td>Olivary dysplasia</td>
<td>[126,127]</td>
</tr>
<tr>
<td></td>
<td>Olivary dysplasia</td>
<td>[117,119]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>Increased cell density and reduced neuronal size</td>
<td>[119]</td>
<td>Cytoarchitectonic disturbances</td>
<td>[128]</td>
</tr>
<tr>
<td>Facial nucleus</td>
<td>Cell density decreased by 95%</td>
<td>[120]</td>
<td>Heterotopic neurons</td>
<td>[127,129,130]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Less distinct boundaries</td>
<td></td>
</tr>
<tr>
<td>Hippocampus (CA4 and subiculum)</td>
<td>Increased cell density and reduced neuronal size</td>
<td>[119]</td>
<td>Altered fiber input from entorhinal cortex, cytoarchitectonic disturbances</td>
<td>[131]</td>
</tr>
<tr>
<td></td>
<td>Decreased dendritic branching</td>
<td>[119]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdala (central, medial, cortical nuclei)</td>
<td>Increased cell density and reduced neuronal size</td>
<td>[119]</td>
<td>Cytoarchitectonic disturbances</td>
<td>[128]</td>
</tr>
</tbody>
</table>

*For reviews, see Refs [9, 10, 121].
Role of cerebellar pathology in autism

• Clinical and experimental evidence for non-motor functions of the cerebellum (visuospatial, executive, memory, language, attention, emotion).

• Language, memory and social alterations (autistic syndrome) after cerebellectomy in children.

• Decreased volume of the cerebellar vermis in autistic patients. Decreased number of Purkinje cells in autistic patients (postmortem studies).

• Neuroanatomical studies in twin pairs that are discordant for autism suggest that cerebellar (as opposed to cerebral) anatomy is an important link between genetic liability and behavioural syndrome.

• Genetic syndromes involving congenital cerebellar abnormalities (e.g. Joubert syndrome) are associated with autism only in presence of severe hypoplasia of cerebellar hemispheres and/or vermis.
Role of cerebellar pathology in autism

- Structural alterations of the cerebellum in autism probably reflect alterations of a network
- 1) the cerebellum receives input from nearly all neural systems,
- 2) sends outputs to nearly all neural structures, and
- 3) has a stereotyped organization that is highly conserved among mammals, it may be a particularly sensitive *indicator* of broad developmental alterations that affect several neural systems at once.

*Animal models with developmental alterations affecting several neural systems, including the cerebellum, may be a useful model for disease mechanisms in autism.*
The reeler mouse

- Spontaneous genetic mutation
- Autosomic recessive mutation
- Defects shown in the development of the cerebral cortex (inversion) and of the cerebellum (cerebelar hypoplasia)
- Hypotonic, loss of motor coordination, (reeling), high frequency tremor
- Behavioural abnormalities
- The gene product is called Reelin (extracellular matrix protein)
Why the Reeler heterozygous (rl/+)
mouse?

- The homozygous Reeler mouse (Knockout) shows abnormal CNS organization.
- Reelin is 50% reduced in the rl/+ mouse cerebellum.
- The male rl/+ mouse has a reduction of PC number.
Photographs of dissected cerebella from heterozygous control (A) and homozygous reeler (B) mice. (A) The cerebellar lobules apparent from a dorsal viewpoint are indicated. (B) The reeler cerebellum is greatly reduced in size and lacks foliation. The borders between vermis and hemispheres are indistinct. The pfl/fl are joined in a poorly developed complex. Roman numerals in A indicate individual, normal lobules. Superior colliculus, sc; inferior colliculus, ic; simplex lobule, sim; paramedian lobule, pm; copula, cop; paraflocculus, pfl; fused paraflocculus and flocculus, pfl/fl. Scale bar 1 mm. E. Ilijic et al. / Neuroscience 136 (2005) 633–647 635
Ultrasound vocalisations

7-day-old male and female mice from three genotypes:
- Wild type: Wt
- Heterozygous: Hz
- Reeler: Rl

from two perinatal treatments:
- Handled: H
- Separated: Sep

Ognibene E, W Adriani, S. Macrì, G Laviola
Amphetamine-stimulated locomotion minus baseline level (left panel) and duration (s) of stereotyped behavior (right panel).

Mice were injected with the drug (10 mg/kg) and returned to the apparatus. A marked Reln-gene dose-dependency was shown.

Amphetamine-stimulated locomotion minus baseline level (left panel) and duration (s) of stereotyped behavior (right panel). Mice were injected with the drug (10 mg/kg) and returned to the apparatus. A marked Reln-gene dose-dependency was shown by VEH-exposed controls, and such Reln-gene dose-dependency was redirected quite in an opposite direction as a consequence of prenatal CPF-O exposure.

PAIN THRESHOLD and ANALGESIC RESPONSE TO MORPHINE (10 mg/kg).

° Panel A: Tail-flick latency at several time points following the drug injection

° Panel B: Density of opioid mu-receptors in the mid-brain.

Male PCs seem to be more sensitive than female PCs to Reelin haplo-insufficiency: is this a model of increased vulnerability of the developing male brain for genetic insults? (increased prevalence of autism & ADHD in males)

**Mechanism:**

a) fetal/neonatal effect of gonadal steroids; b) neuroprotective gene on X chromosome
Theories accounting for the male prevalence of ASD: The existence of a sex bias in ASD, with a male to female sex ratio of 4:1 for autism and 8:1 for AS, have led some researchers to link autism to “maleness”.

A very intriguing theory, developed by Baron-Cohen et al. Considers autism as an “extreme form of male brain”. According to the Extreme Male Brain Theory, in fact, autistic individuals show an extreme pattern of the typical male brain functions.

This theory moves its steps from the clear evidence for a sexual dimorphism both in neuropsychological features and in brain neurobiology. Indeed, women are reported to be better on average at verbal and social tasks, showing stronger ability in “empathy”, while men as a group are superior at “systemizing”.


The key mental domains in which sex differences have traditionally been studied are verbal and spatial abilities. In this article I suggest that two neglected dimensions for understanding human sex differences are 'empathising' and 'systemising'.

The male brain is defined psychometrically as those individuals in whom systemising is significantly better than empathising, and

the female brain is defined as the opposite cognitive profile. Using these definitions, autism can be considered as an extreme of the normal male profile.
Autism and male rl/+ mouse

• Reduction of PC number

• Levels of Reelin in the +/rl cerebellum is 50%

• Male sex is a risk factor
Behavioral disorders of development

Polymorphisms of other genes

Non-genetic factors (gonadal steroids, endocrine disrupters, antibodies, infective agents, neurotoxins, "chance")

Modifying factors

Gene function

Full mutation

- 100% function
  - Normal development

- Polymorphisms
  - Behavioral disorders of development

- Full mutation
  - Severe neurological deficits

Non-genetic factors (gonadal steroids, endocrine disrupters, antibodies, infective agents, neurotoxins, "chance")
Modifying factors

Polymorphisms of other genes

Non-genetic factors (gonadal steroids, endocrine disrupters, antibodies, infective agents, neurotoxins, “chance”)

Full mutation
- 100% function
  - Severe neurological deficits
  - Normal development
  - Behavioral disorders of development

Reln function
Numbers of PC adult in +/- and rl/+ mice

from Hadj-Sahraoui et al., J. Neurogen. 11:45-58, 1996
Total number of Purkinje cells in cerebella of neonatal (P10-P18) heterozygous *reeler* mice

Effects of neuroactive steroids and Reelin gene dosage on Purkinje cell survival during development
Biamonte F. et al, and F. Keller (2009) *submitted*
Testosterone (panel A) and 17 beta estradiol (panel B) levels in cerebellum of 5 days old male and female HZ reeler and WT mice

Cerebellum of 5-days old male HZ mice:
° shows a significant increase of testosterone levels
° a simultaneous decrease of 17 beta estradiol.
° This is not evident in case of female.

Effects of neuroactive steroids and Reelin gene dosage on Purkinje cell survival during development
Effects of neuroactive steroids and Reelin gene dosage on Purkinje cell survival during development
• $17\beta$-Estradiol increases Purkinje cell number in males rl/+ mice.

• The results by Biamonte and Keller suggest that both Reelin and estrogens may converge to promote the survival of PCs during critical periods of postnatal cerebellar development.

• These results are clinically interesting because several neurodevelopmental disorders, particularly autism spectrum disorders, show an increased prevalence in males and a decreased number of PCs in the cerebellum.

• It is interesting that the cerebellum of P5 male RL/+ mice shows a significant increase of testosterone levels and a simultaneous decrease of $17\beta$ estradiol.
**Homing test:** early social motivation. On pnd 9, pups have to reach the nest area in a T-maze apparatus by making a choice for the nest odor.

- A significant lower percentage of rl/+ mice reached the nest area than corresponding wt pups.
- In the absence of motor changes, this indicates a genotype-dependent alteration in sensitivity to or in central processing of social stimuli.
- Remarkably, this deficient profile was reverted by neonatal estradiol.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>HZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>33</td>
<td>19.3*</td>
</tr>
<tr>
<td>ESTR</td>
<td>19.4</td>
<td>37.5**</td>
</tr>
</tbody>
</table>
Autism is a neurodevelopmental disorder that is behaviourally defined by three core symptoms:

° Deficits in reciprocal social interaction

° Qualitative impairments in social communication

° Stereotypies, repetitive, ritualistic behaviors, restricted interests
Example of a complete IDED task

<table>
<thead>
<tr>
<th>Stage</th>
<th>Rewarded stimuli</th>
<th>Discrimination 1</th>
<th>Discrimination 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple discrimination SD</td>
<td>Sawdust</td>
<td>Sawdust VS Cotton</td>
<td></td>
</tr>
<tr>
<td>Compound discrimination CD</td>
<td>Sawdust</td>
<td>Sawdust + Cinnamon VS Cotton + Sage</td>
<td>Sawdust + Sage VS Cotton + Cinnamon</td>
</tr>
<tr>
<td>Intra-Dimensional shift IDS</td>
<td>Shredded Paper</td>
<td>S. Paper + Oregano VS Confetti + Thymus</td>
<td>S. Paper + Thymus VS Confetti + Oregano</td>
</tr>
<tr>
<td>Extra-Dimensional shift EDS</td>
<td>Parsley</td>
<td>Tissue + Parsley VS Styrofoam + Anise</td>
<td>Tissue + Anise VS Styrofoam + Parsley</td>
</tr>
</tbody>
</table>
Neuropsychological Tests

Wisconsin Card Sorting Test

- Widely used in humans
- Assess the capacity to shift between cognitive attentional sets
- Determine / quantify cognitive impairment in psychological pathologies and in patients with prefrontal cortex lesions

Need of language and abstract concepts

Adaptation of this human task to allow the use in rodents

Intra-dimensional Extra-dimensional set shifting task

Discriminations involving compound somatosensory and olfactory stimuli

Stimuli vary in independent “dimensions”

- odor
- digging medium

Only one stimulus is the cue to the reward in each stage

Animals with brain lesions or dysfunction have higher difficulty in reaching the criterion to each stage
Adult male mice were assessed in an attentional set-shifting task (Colacicco et al., 2002), involving the formation of new rules to obtain a palatable reward. rl/+ subjects showed a higher number of perseverative responses. Neonatal estradiol contrasted this profile.

**Attentional set-shifting test**

Bar graph showing number (± SEM) of trials to criterion (8 consecutive correct trials) for each discrimination. Male mice significantly differed in the CDR task, as a function of genotype and early hormonal treatment. In particular, Dmso-injected wt mice learned CDR significantly quicker compared to rl/+ controls. Estradiol improved significantly the performance in rl/+ mice.

wc: waiting compartment; cc: choice compartment; fc: food cup.
Molecular mechanisms

Reelin
GABA
5-HT

Anatomic functional alterations

Cerebellum
Amigdala

Clinical syndrome

Altered social intelligence
Behavioral disorders of development

Polymorphisms of other genes

Non-genetic factors (gonadal steroids, endocrine disrupters, antibodies, infective agents, neurotoxins, “chance”)

Modifying factors

Full mutation

Polymorphisms

100% function

Gene function

Severe neurological deficits

Behavioral disorders of development

Normal development
Walter Adriani
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