CANDY

Comorbid Analysis of Neurodevelopmental Disorders and epilepsY

Deliverable D2.6

Protocol on translational behavioral and brain excitation/ inhibition measures for exploitation

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Table of Contents

1 Executive summary

This document describes Deliverable 2.6, involving a protocol for 1. the translational test battery for mice, and 2. The measurement of excitation/inhibition balance in mice.

The test battery addresses the six bio-behavioural domains that play a central role in CANDY: 1) social communication/social processing, 2) reward processing, 3) emotional reactivity, 4) predictability, 5) sensory processing, and 6) attention. These six biobehavioural domains are affected in neurodevelopmental disorders and their comorbidities and are measured in WP2 both in mice and in children. We describe the protocols for the tests that measure each of these domains.

The central hypothesis of the CANDY project is that the comorbidity between neurodevelopmental disorders and epilepsy converges on changes in the excitation / inhibition balance. The measurement of the excitation/inhibition balance is done at two levels: ex vivo, using western blotting, and in vivo, using magnetic resonance spectroscopic (MRS) imaging. We describe the protocols for both procedures.

2 Introduction

The CANDY project aims to understand the mechanisms underlying neurodevelopmental disorders and their comorbidities along six biobehavioural domains: 1) social communication/social processing, 2) reward processing, 3) emotional reactivity, 4) predictability, 5) sensory processing, and 6) attention. These biobehavioural domains serve as constructs to get closer to the understanding of the behavioural mechanisms than clinical diagnoses. These biobehavioural domains are measured in children with neurodevelopmental disorders as well as animals and serve as a way to bridge human and animal research. The behavioural test battery has been set up such that it is maximally aligned with the PIP Tablet tasks for children. This alignment is based on regular contact between the 'animal' and 'human' researchers in WP2. For instance, the social reward test has been specifically designed for the children based on the task earlier developed for animals. The attention task based on principles of reinforcement learning are also carefully aligned between the animals and humans, ensuring that the same concepts are being measured. Nonetheless, mice are not humans, and therefore differences between the two species cannot be fully avoided. For instance, in children emotionality is measured using an emotion recognition test, which cannot be applied as such to animals. In animals we instead use the widely used and well validated elevated plus maze test. To measure predictability, a validated predictability test is used where the time the mice need to habituate to a new environment while being exposed to predictable and unpredictable sounds is being measured. In humans, no such a test exists, but instead the children's ability to detect coherent motion of objects is being measured. Sensory processing is measured in mice using the prepulse inhibition test. While this test can be employed in humans, it is less suitable for young children because loud noises are used. Instead, children are tested for tactile and auditory processing. Social behaviour in mice is measured by assessing social interaction and vocalizations, which is their way of communication. While these measures can also be taken from children, such measures cannot be implemented in tablet format. Instead, in children gaze following social stimuli are measured.

The hypothesis of the CANDY project is that the comorbidity between neurodevelopmental disorders and epilepsy relates to a change in the excitation / inhibition balance. To measure the excitation / inhibition balance we have undertaken two types of measurements: 1. Ex vivo analyses (Western blotting) of glutamate system components (involved in excitation) and 2. In vivo analyses (Magnetic Resonance Spectroscopy; MRS) of brain metabolites. While the ex vivo analyses can exclusively be conducted in experimental animals (as it is ethically not allowed to take brain biopsies from children), the in vivo analyses can be conducted both in animals and humans.

In the next section we describe the behavioural test battery for mice in more detail, as well as the Western blot and MRS procedures. These descriptions are based on the animal ethics permit where the methods are described in detail.

3 Main section

3.1 Translational test battery for mice

3.1.1 Description of the translational test battery protocol for mice

The translational test battery for mice aims to cover six biobehavioural domains:

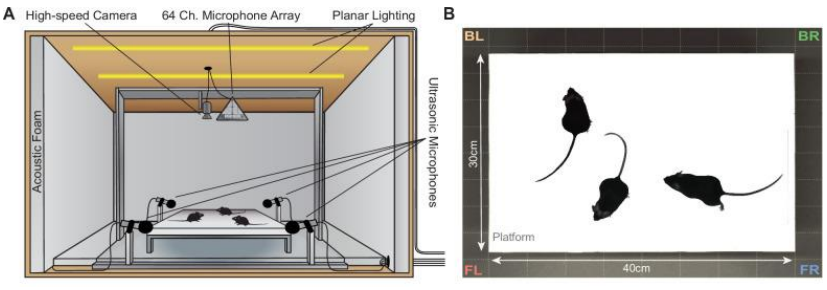
1) social communication/social processing [group 1]
2) reward processing [group 1]
3) emotional reactivity [group 1]
4) predictability [group 1]
5) sensory processing [group 1]
6) attention [group 2]

To test mice along these six biobehavioural domains, at two ages (adolescence and adulthood), the tests for these six domains are divided over three groups of mice. The reason is that adolescence is a short period in mice, taking place between postnatal day 30 and 60, meaning that the behavioural tests must be conducted within this period to tackle the adolescent age. Domains 1-5 can be tackled by relatively short tests and therefore can be grouped together. Domain 6 takes 3-4 weeks of training and testing, and thereby is tested in a separate group of mice.

The tests are not containing stressors. Nonetheless, the prepulse inhibition test makes use of loud sounds to induce startle responses. To avoid carry over effects from this test to other tests, the pre-pulse inhibition is planned as the last task in the test battery.

Group 1

Social communication – 1 x 10 min and 2 x 10 min



Mice produce ultrasonic vocalization to communicate their emotions and intentions. Shank3 knockout mice show changes in ultrasonic vocalizations compared to wild-type mice during social interactions [1]. However, it was so far not possible to determine who was calling in this previous work. With a new tool this is now possible to localize ultrasonic vocalizations, and thereby to determine which animal is calling in social interactions [2]. We apply this new tool to measure

ultrasonic vocalizations in male mice. Male mice vocalize when meeting a female. We measure ultrasonic vocalizations during triads of social interactions, in which 2 male mice meet a female mouse, to assess whether the vocalizations of the males are dependent on the social context (i.e. the genotype of the other male). All mice are unfamiliar to each other. First, the mice are habituated to the test arena (soundproof booth) for 10 min. Then, the males and females are placed together for 10 min. The males are tested at max. in three different genotype configurations. The females are always wildtype. Only adult mice are tested, being sexually fully matured. We have successfully applied this regime before, based on which we can state that the 10 min under light conditions will be too short and unsuitable for sexual intercourse, and that the male mice are also not aggressive to each other under these conditions [2]. Social communication is tested in a separate group (group 0), since the experimental conditions (testing under light conditions) and factors (age, sex) are different for group 0 compared to group 1 and 2.

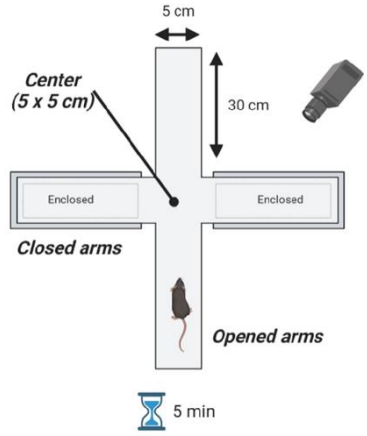
Social processing – 2 x 10 min and 1x 15 min:

To assess social processing mice are first habituated to the test cages for 2 x 10 minutes. Prior to testing the mice are socially isolated for 3 hours, to induce the motivation of the mice to socially interact. Then, the animals are allowed to socially interact for 15 min in a large arena with 3-4 other unfamiliar conspecifics of varying genotypes [3].

Social reward – 5 days:

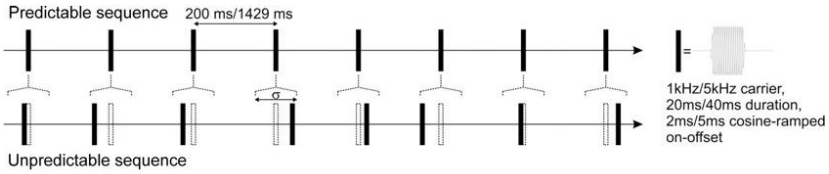
An experimental cage similar to the animal's home cage is used for this task. The bedding is replaced after each trial and water and food is available. During the habituation phase (4 days), mice are placed in the cage with a nonfamiliar conspecific. The animals are let free to explore the cage and to interact with each other for 15 min. At the end of the trial, the experimental and stimulus mice are returned to their homecage. For four consecutive days, the experimental mouse is exposed to the same conspecific and habituated to the environment and the social stimulus. On day 5 the novelty phase takes place. A nonfamiliar conspecific is placed with the experimental mouse in the cage for 15 min to allow direct interaction. In total, the experimental mice are exposed to two different conspecifics: one social stimulus repeatedly presented from day 1–4 (habituation phase) and a second mouse at day 5 (novelty phase). During the social habituation/nonfamiliar exploration task, nonaggressive interaction is scored (experimenter blind to genotype group) when the experimental mouse initiates the action and when the nose of the animal is oriented toward the social stimulus mouse only. The time interaction is used to calculate a Novelty Index as follows: InteractionDay 5 − InteractionDay 4.

Emotional reactivity – elevated plus maze 5 min/light spot test 3 days + 1 hour:

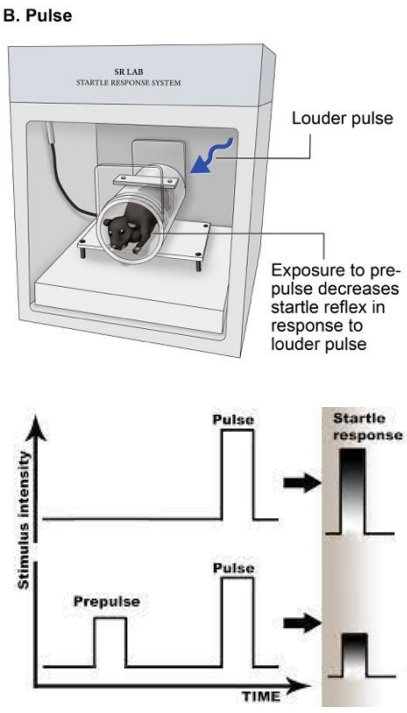


The test device is elevated from the floor with two open arms (30 cm length × 5 cm width) and two enclosed arms (30 cm length × 5 cm width × 10 cm height) with a square central cross connection area (5 cm × 5 cm). The animal is placed in the center of the maze, facing one of the open arms, for a free exploration period of 5 min. The movements and position of the animals are automatically recorded and measured using EthoVision XT 10 software (Noldus Information Technology, Wageningen, The Netherlands). Results are expressed as the mean of time spent (s) in open arms, the mean time spent in closed arms, and the distance moving (cm) in both open and closed arms [4].

Predictability -5-10 min:



To assess the ability to predict events, the habituation of mice to unpredictable **non-aversive** auditory tones in a novel environment (open field; elevated plus maze) is measured [5]. To avoid repeated testing effects, a plus-maze is used with a different context than the elevated plus-maze that is used to measure emotionality. The tests take 10 (open field) and 5 (elevated plus maze) minutes. Primary readout: Open field: General locomotor activity during sound pulse sequence stimulation. Elevated plus-maze: The time spent (s) in the different compartments (closed vs open arms).



Sensory processing – prepulse inhibition 30 min:

Mice are placed in a Plexiglas tube resting on a plastic frame. A piezoelectric accelerometer mounted under the tube detected and transduced the motion of the tube. Throughout the startle session, a background noise of 70 dB is maintained. The experiment starts with a 5 min habituation session with background noise in the startle system. After this habituation period, ten blocks of five trials are delivered to measure prepulse inhibition. Each of these blocks consists of one startle trial (110 dB, 20 ms broad band burst), one no-stimulus condition, and three different prepulse-startle pairings administered pseudorandomly. In these pairings, the prepulse was 3, 5, or 10 dB above background and always 20 ms broadband burst followed by the 110 dB startle pulse 100 ms later. The interval between two trials is between 10 and 20 s. The startle amplitude is calculated as the mean of 10 delivered startle trials. The degree of prepulse inhibition (in percentage) is calculated according to the formula: 100 – (Average of startle amplitude on prepulse trial/ Average of startle amplitude on startle trial) × 100 [4].

Group 2

Attention / executive functioning – 3-4 weeks (1 session of 30-60 min per day):

Mice are tested in touchscreen operant boxes. These consist of operant arenas housed within dense fibreboard sound-attenuating chambers that are equipped with a fan to provide ventilation and mask background noise. The operant arenas consist of a perforated stainless-steel floor enclosed by trapezoidal walls opening onto the touchscreen (12.1 in.; resolution 800 × 600). The touchscreen is equipped with infra-red (IR) beam arrays positioned less than 5 mm from the surface of the screen such that animals do not have to apply pressure directly to the screen for a response to be detected. A reward collection magazine connected to a pellet dispenser is attached to the wall opposite the touchscreen to deliver 20-mg reward pellets. To test attention, a visual detection task is used in which a visual stimulus is presented randomly on either the left or right side of a touchscreen operant box. Correct responding to the stimulus is rewarded by a pellet. In correct responding is punished by a time-out period of 10 seconds. The mice are tested for 1 session a day lasting 30-60 min, and approximately 3-4 weeks in total (depending on how fast the animals learn). The task is considered complete when the animals reach the criterion of 80% correct responding.

To motivate the animals for the attention task, it is essential to give the animals a reward. Such a reward has only value when it fills a motivational need. To this end the animals are food deprived up to 85-90% of their free-feeding body weight. For each genetic mouse line, a growth curve is generated to determine the reference weight and subsequently calculate the percentage of body weight loss in the food-restricted animals. The animals are weighed daily during weekdays, just before they are reunited with a peer. Additionally, the food intake of the animals is monitored by weighing the food in the roof once every two days. To achieve the 85-90% of base weight, mice are restricted to 70% normal intake. A normal adult mouse has an approximate intake of 3-6g., therefore they have access to 2-4g. During weekends they have access to double the food available during the weekdays. The food intake of the animals is weighed 1-2 weeks prior to food restriction to obtain information about the free feeding food intake and to determine the amount of food that should be given to the animals. Food will be allocated based on the average weight of a pellet, and the use of split pellets. Because of competition between socially housed animals when food is limited, the animals are socially isolated. Yet, the mice are allowed to social interact each day for 2-3 hours before being separated again and receiving their daily portion of food.

3.1.2 Description of the measurement of the excitation / inhibition balance in mice

1. Ex vivo measurement of components of the glutamate system in mice.

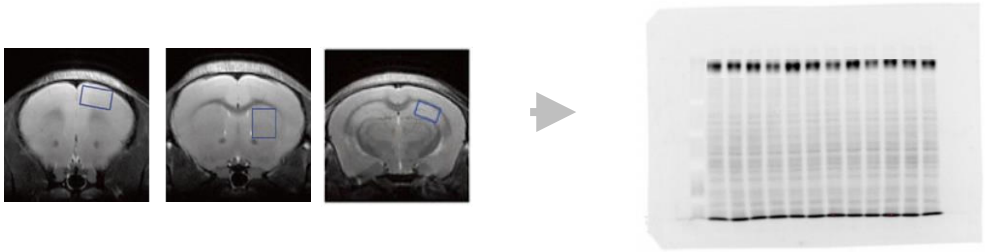
Mice are killed by through decapitation without anesthesia at adolescence (P30) or adulthood (P70).

No anaesthesia is given, because anaesthesia can confound gene expression. The brains are immediately removed from the skulls and snap frozen on dry ice. These brains are subsequently stored at -80 degrees.

Synaptosome extraction

Brains stored at -80°C are gradually brought to -20°C over 24 hours. Using a cryostat, the brains are cut in 200µm-thickness slices, and mounted onto glass slides to localize regions of interest like the striatum and hippocampus. These regions are meticulously identified following the Paxinos and Franklin's mouse brain atlas, and carefully extracted using biopsy pens.

For synaptosome extraction, tissue weights are crucial for determining the appropriate volume of Syn-PER™, a reagent needed for isolating synaptosome fractions. For every milligram of tissue, 100µl of Syn-PER reagent containing 1X Thermo Scientific™ Halt™ Protease Inhibitor Cocktail (100X) is added. A battery-operated homogenizer is employed to homogenize the tissue, followed by centrifugation at 1200x g for 10 minutes at 4°C to remove cell debris. The resulting supernatant is collected and subjected to further centrifugation at 15000x g for 20 minutes at 4°C to pellet the synaptosomes. The cytosolic fraction (supernatant) is stored separately for potential future experiments. The synaptosome pellet is then resuspended using of Syn-PER reagent and preserved at -80°C following the protein quantification of each sample using the Thermo Scientific™ Micro-BCA Protein Assay Kit.



Western blotting

For Western blotting, a resolving gel (7.5%) and a stacking gel (4%) are prepared for SDS-PAGE. Samples are mixed with sample buffer containing 4% beta-mercaptoethanol to denature proteins by breaking cysteine bonds. The sample buffer volume is doubled and adjusted to a total volume of 15µl with ultrapure water. Samples are heat-shocked for 3 minutes at 100°C before loading onto the

gel, which runs for 30 minutes at 60V followed by approximately 80 minutes at 125V. Prior to blotting, the gel is incubated in running buffer for at least 5 minutes and briefly in ultrapure water.

PVDF membranes are activated with 100% methanol for 10 seconds, followed by incubation in ultrapure water for 5 minutes. Membranes, along with filter paper, are then incubated in 1X Power Blotter Transfer Buffer for at least 5 minutes. In the transfer system, a stack is formed with the gel, membrane, and filter papers, oriented to facilitate migration of negatively charged proteins from the gel to the membrane. Total protein staining is performed using Invitrogen™ No-Stain™ Protein Labeling Reagent for normalization. After blocking in TBS-Tween buffer with 5% non-fat dry milk, primary antibody incubation is conducted overnight at 4°C using the following antibodies against the proteins of interest: anti-GluA1 Recombinant Anti-Glutamate Receptor 1 (AMPA subtype) antibody (ab183797), 1:1000; anti-GluA2 Recombinant Anti-Ionotropic Glutamate receptor 2 antibody (ab206293), 1:1000; anti-GluN1 Recombinant Anti-NMDAR1 antibody (ab109182), 1:500; anti-GluN2a Recombinant Anti-NMDAR2A antibody (ab124913), 1:1000, and anti-GluN2b Recombinant Anti-NMDAR2B antibody (ab183942), 1:1000. Then, the membranes are washed and incubated with secondary antibody before visualization using Amersham™ ECL™ Western Blotting Detection Reagents and imaging with the iBright750 imager.

2. Magnetic Resonance Imaging and Spectroscopy in anaesthetized mice

Animals undergo Magnetic Resonance Imaging, a non-invasive and non-terminal imaging procedure based on the application of non-ionizing radiofrequency waves within a strong static magnetic field. Animals are imaged on systems operating at either 7 Tesla or 11.75 Tesla, with radio frequencies in the range of 300mHz and 500mHz respectively. The energy deposition from the radio frequency do not exceed the specific absorption rate implemented by the machine vendors (Bruker Biospin MRI Gmbh, Ettlingen, Germany), and should not lead to tissue heating. The MRS procedure is performed in the MRI system using a different sequence. The procedure acquires a metabolic spectrum indicative of the relative concentrations of the major metabolites in the brain, allowing the investigation of the chemical profile of defined target volumes. The concentration is analyzed offline using dedicated software (LCmodel) [6].

Animals are briefly anaesthetized with 5% isoflurane in 4:1 air to oxygen mix a designated induction box. Animals will be intubated endotracheally. The animals are moved to an MRI-compatible animal cradle equipped with an air outlet connected to a mechanical ventilator to deliver isoflurane in air:oxygen mix, tooth and ear bars to position the animal. The sedative medetomidine (0.05 mg/kg) and muscle relaxant pancuronium bromide (0.4 mg/kg) are administered subcutaneously.

After the positioning of the animal, a breathing pillow is placed under the animal belly to record breathing and body motion, and a radio frequency coil is positioned in contact with the mouse head and connected to the MRI system.

Animal preparation for the experiment is expected to take up to 30 minutes, following which, isoflurane is reduced to 1-0.5% and the cradle is inserted by sliding it on a rail into the magnet bore. MRI recordings in individual animals does not exceed sessions of 3 hours but are typically expected to last 1 hour and 30 minutes. At the end of the MRI session, animals are placed on a warmed recovery station, with 3-1% isoflurane in 4:1 air to oxygen mix being delivered via the mechanical ventilation until the animals show reflexes and can be extubated safely. The animal is put into a recovery box until it becomes responsive, before being returned to its cage.

4 Conclusions

This deliverable provides an overview of the protocols for a translational test battery and the measurement of the excitation / inhibition balance in the brain of mice. This protocol can help others to replicate our studies.

5 Glossary

Abbreviation / acronym	Description
MRS	Magnetic Resonance Spectroscopy
MRI	Magnetic Resonance Imaging
P	Postnatal day
dB	decibel
g	grams

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