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Study Title: Neuroprotective Potential of Isradipine (ISR) Administered via Subcutaneously Implanted Pellets to Alpha-Synuclein (A53T) Transgenic (Tg) Mice – Efficacy Study

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Estimated Study Dates (In-Life): November 19, 2010 to July 31, 2011

**RTI International is the trade name for Research Triangle Institute*

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1.0 ESTIMATED TIMELINE

- Animal receipt 9/28/10; pre-dosing baseline starts 11/19/10; dosing starts 11/22/10.

- Pre-study: Minimum one-week quarantine and acclimation to study room. Individual animal identification (tail marking). Animals assigned to treatment start dates according to age such that treatment starts at ~8 months of age.
- Pre-dosing baseline: Within three days prior to each treatment start date, record body weight, and detailed functional observations, including neurological signs.
- Assignment to Treatment Groups: Within three days prior to each treatment start date, assign animals (i.e., for that treatment start date) to treatment groups (i.e., vehicle or isradipine) by stratified randomization on body weight within sex. Review the clinical status of animals assigned to the two treatment groups (i.e., functional observations and neurological signs) and adjust group assignments if necessary so that groups are as closely matched as possible for body weight and clinical status at the start of treatment.
- On the assigned start date for dosing, each animal will be implanted subcutaneously with a biodegradable pellet containing isradipine or with a placebo pellet. Pellets are designed for continuous drug delivery across a 90-day period and thus a new pellet (isradipine or placebo) will be implanted at 90-day intervals. Starting on the day of pellet implantation, clinical observations (morning) and cage-side checks (afternoon) will be completed and findings recorded. Implantation of pellets at 90-day intervals and daily clinical observations and cage-side checks continue throughout the life-span of each mouse, i.e., until (a) found dead, (b) terminated with approval of the study coordinator, study director and/or facility veterinarian based on clinical criteria, or (c) otherwise scheduled for termination with approval of the study director.
- Regularly Scheduled Observations:
 - Every 90 days: subcutaneous pellet implantation.
 - Once daily (morning): clinical observations.
 - Once daily (afternoon): cage-side checks
 - Weekly: body weights and detailed functional observations, including neurological signs.
- Necropsy: Moribund (unscheduled) and terminal (scheduled) sacs for selected animals, either perfused or non-perfused (include general necropsy if non-perfused). Neural tissues collected, preserved (fixed or frozen) and shipped to University of Minnesota for neuropathological (fixed) or neurochemical (frozen) evaluation [Note: MK Lee consultant; assays at UMn funded separately].
- Save tail snips for genotyping from every animal on study whether found dead or terminated (scheduled or unscheduled) so that genotype can be verified for each animal.
- Study completion is estimated in June-July 2011 based on estimated life span, but may continue longer if the drug treatment improves clinical outcome and life span.

2.0 EXPERIMENTAL SUBJECTS

Transgenic (Tg) males (R9 and R10) were among the live born offspring produced from a rederivation project at Taconic (Project No. 009289) and these two males sired the next generation of offspring during colony expansion. Counting Tg males R9 and R10 as the first generation, the third generation offspring will be used for this study. [Note: Tg donor males (alpha-synuclein A53T mice) for the rederivation were provided by MK Lee, Johns Hopkins University].

All study animals received a unique individual animal identification number and an ear punch code at Taconic. Following receipt at RTI, the unique individual animal number will be marked on the tail of each animal with indelible ink.

While at Taconic, tail biopsies were collected and shipped to RTI for genotyping. Records of tail sample receipt and genotyping results will be maintained in the study records and carefully cross checked with the genotype recorded on the shipping inventory for mice arriving from Taconic. Furthermore, after the animals are received at RTI, the genotype will be verified based on tail snips to be collected when the animal is found dead or terminated (regardless of the cause of death or reason for termination).

Date of birth (DOB) for study animals is reported as the week in which the mouse was born, such that DOB = Week 0). Animals designated for this study were born during the weeks of March 29, 2010 through May 31, 2010. Mice were shipped from Taconic to RTI and received on September 28, 2010. On a weekly basis, the oldest mice (i.e., earliest DOB) will be assigned to study until all treatment groups are filled. Thus, ~55 Tg mice will be assigned to study (27 or 28 per treatment group), and dosing for individual animals will begin at ~8 months of age, with estimated life span of ~12-14 months.

Alpha-synuclein (A53T) mice display a progressive phenotype that includes abnormal behavior, neurological decline and early mortality ([Lee et al., 2002](#); [Smith et al., 2010](#)). The behavioral phenotype and indices of neuropathology appear normal through ~4 months of age. In the preclinical phase (~5-7 months of age), abnormal behavior includes hyperactivity (horizontal and vertical), sustained posturing, bradykinesia, mild ataxia and dystonia. In the later clinical stages (>8 months), mice develop progressive movement dysfunction leading to paralysis and death ([Lee et al., 2002](#); [Smith et al., 2010](#)). To be more specific, another laboratory reported onset of clinical symptoms at ~8 months of age with ~50% survival of A53T mice at ~9 months of age (263 ± 10 days; Smith et al. 2010). In the absence of pharmacological intervention, none of the A53T mice survived beyond ~13 months of age ([Smith et al., 2010](#)).

3.0 SENTINELS AND ANIMAL HEALTH SURVEILLANCE

Commercially available young adult C57Bl/6 mice have already been purchased as sentinels for the study room and were received from Taconic on the same date as the study animals (September 28, 2010). Health surveillance screening will follow RTI Animal Research Facility (ARF) Standard Operating Procedures (SOPs). Sentinels will be scheduled for specimen collection after ~4 weeks of residency in the study room and at ~12 week intervals thereafter until the end of the study.

4.0 ANIMAL HUSBANDRY

4.1 Animal Housing and Environmental Enrichment

Study animals will be housed in the study room upon receipt. Animals for more than one pharmacology study may be housed in the same room (i.e., while awaiting study start or while actually on study). Study animals will be singly housed in solid-bottom polycarbonate cages (5" x 11.5" x 7") with water bottles and certified Irradiated Sani-Chips[®] cage litter from P.J. Murphy Forest Products. Copies of the bedding certification will be maintained in the study record book. Assigned personnel will change cages at least weekly according to ARF SOPs.

Autoclaved Nestlets[™] (Ancare, Bellmore, NY) will be furnished in all cages. Feed will be placed in the bedding for food foraging, which is another form of environmental enrichment (see Section 4.3 below). Other forms of physical manipulanda may be introduced into the cage as long as all animals have equal access to the same type of environmental enrichment.

4.2 Environmental Conditions

Environmental conditions will be continuously monitored, controlled, and recorded (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal[®] Software, Siebe Environmental Controls, [SEC]/Barber-Colman Company, Loves Park, IL). Animal rooms will be maintained on a 12:12 hour light:dark cycle. Set points and target ranges for temperature (°F) and relative humidity (RH) in the animal rooms were 72°F (69-75°F) and 50% RH (35-65% RH), respectively.

4.3 Feed

Rodent NIH-31 Modified Autoclavable diet (Zeigler Bros, Inc., Gardeners, PA) will be available *ad libitum*. [Note: RTI will purchase the diet through Taconic Farms, Germantown, NY and the diet will be autoclaved at Taconic prior to shipping to RTI]. Feed will be provided to mice through cage lid hoppers and scattered in bedding to promote foraging (a form of environmental enrichment). If an animal is debilitated (i.e., no longer foraging and/or unable to reach the feed hopper), feed will be moistened and placed within easy access on the cage floor and/or Nutra-Gel Diet (Bio-Serv Inc.) may be placed in the cage to supplement feed/water intake. Nutra-Gel Diet may be used at the discretion of the study staff and/or under direction of the facility veterinarian. Batch numbers and expiration dates for diet will be recorded per RTI SOP. Certified analysis of each NIH-31 feed lot will be provided by the manufacturer and included in the study records.

4.4 Water

Tap water from the Durham, NC, municipal water system will be available *ad libitum* and delivered via water bottles. Water bottles will be changed at least weekly. The city of Durham analyzes drinking water on a monthly basis for potential drinking water contaminants and issues the results annually. A copy of each relevant annual report will be maintained in the study records. Any other relevant results of animal drinking water analyses (i.e., samples collected in the RTI Animal Research Facility) will be maintained in the facility records. As noted above ([Section 4.3](#)), Nutra-Gel Diet (Bio-Serv Inc.) may be used to supplement feed/water intake as warranted by the condition of an individual animal; batch numbers and expiration dates will be recorded per RTI SOP.

4.5 Animal Identification

At Taconic, these mice were individually identified by ear punch and given a unique animal identification number. On arrival, individual animal identity was traceable by ear punch code, packing crate number and packing crate section. A copy of the ear punch code will be maintained in the study room and otherwise available to all study personnel. To assure continuity of individual animal identification, mice be given a tail marking (indelible marker and/or tail tattoo) after arrival at RTI and the tail number will be the same as the unique animal identification number assigned by Taconic.

4.6 Standards for Animal Care

RTI's Animal Research Facility (ARF) is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and operated in conformance with the Guide for Care and Use of Laboratory Animals (NRC, 1996). Animal use for this study was reviewed and approved by RTI's Institutional Animal Care and Use Committee (IACUC) prior to study initiation.

5.0 EXPERIMENTAL DESIGN AND TREATMENT

5.1 Treatment Groups

Animals will be assigned to treatment start dates according to DOB such that treatment begins at ~8 months of age for each animal. Within three days prior to each treatment start date, animals for that treatment start date will be assigned to treatment groups (i.e., vehicle or isradipine) by stratified randomization on body weight within sex. Clinical status of animals assigned to the two treatment groups (i.e., functional observations and neurological signs) will be reviewed and group assignments adjusted if necessary so that groups are as closely matched as possible for body weight and clinical status at the start of treatment.

In general, baseline (pre-dose) measurements will be taken on a Friday and dosing (pellet implant) will be initiated on the following Monday (i.e., within three days). If occasional exceptions occur (e.g., due to unavoidable scheduling conflicts), these cases will be documented in the study records.

This process will continue until both treatment groups are filled with approximately equal numbers of animals in each group with a minimum of 15 mice per treatment group X 2 treatment groups = ~30 mice on study. Both males and females will be represented within each treatment group, and if possible, the ratio of males to females will be approximately the same for each treatment group. [Note: Based on the available population of transgenic mice at study initiation, the maximum will be ~27-28 mice per treatment group X 2 treatment groups = ~55 mice on study].

5.2 Test Drug, Vehicle and Dose Formulation

Test Drug: Isradipine (CAS No. 75695-93-1) will be purchased by Innovative Research of America, Sarasota, FL. Purity of >99% for the bulk drug is anticipated. Source (vendor), lot number(s) and batch number(s), as well as a copy of the vendor's Certificate of Analysis for the bulk drug will be requested and maintained with the study records. Ideally, one lot/batch of bulk

drug will be used for the entire study. Additional bulk drug of comparable purity from the same source may be purchased if necessary to complete the study.

Vehicle: Placebo pellets (90-day), Innovative Research of America, Sarasota, FL.

Formulation: Custom pellets for delivery of isradipine will be purchased from Innovative Research of America, Sarasota, FL. Dose delivery characteristics of isradipine pellets will be defined in a custom order, taking into consideration the projected body weights of mice at start of dosing, and with the goal of administering 5 mg/kg/day isradipine continuously through uniform release throughout the period of drug administration. Historical data for body weight in this transgenic line of mice will be used to determine any adjustments in characteristics of the drug delivery system as the animals age with the goal of maintaining delivery as close as possible to the nominal drug dose.

Procurement and Records: Biodegradable pellets (isradipine and placebo) will be procured, received and stored by RTI Materials Handling Facility (MHF). Verified copies of records [i.e., receipt and disposition, vendor's certificate of analysis (COA) for the bulk drug, Material Safety Data Sheet (MSDS), dose formulation, inventory and use logs] will be maintained for the study records.

Storage and Expiration Dates: For implantable pellets (isradipine and placebo), the vendor's recommended storage conditions and expiration dates will be observed. Specifically, refrigerated (~4°C) and protected from light and moisture.

Safety: A copy of the MSDS for isradipine will be maintained in all active work areas where the pellets are stored or in use. Procedures recommended in the MSDS for bulk chemical handling will be followed. Routine safety precautions, including PPE appropriate to the work area, will be followed for handling pellets.

Disposition of Bulk Drug and Dose Formulations: Any residual pellets (isradipine or placebo) will be maintained in the MHF until disposition is approved by the Study Director. In the absence of specific instructions, the default disposal date will be not sooner than 30 days after the pellet expiration date or not sooner than 30 days prior to completion date of the referenced prime contract (whichever comes first). Residual pellets (isradipine or placebo) will be processed as waste for incineration, unless otherwise instructed by the Study Director.

5.3 Treatment Regimen

Treatment groups will have assigned Color Codes (Table 1) to facilitate accuracy of dosing and clarity of study records.

On the assigned start date for dosing, each animal will be implanted subcutaneously with a biodegradable pellet containing isradipine (treated group) or with a placebo pellet (vehicle control group). Pellets will be selected for continuous drug delivery at a uniform rate across a 90-day period of drug exposure. Thus, for each mouse a new pellet (isradipine or placebo) will be implanted at 90-day intervals throughout the life-span of each mouse, i.e., until (a) found dead, (b) terminated with approval of the study coordinator, study director and/or facility veterinarian based on clinical criteria, or (c) otherwise scheduled for termination with approval of the study director.

Each pellet will be weighed and then implanted using an appropriately sized trocar (Innovative Research of America, Sarasota, FL). Pellets will be placed by puncturing the skin between the scapulae with the trocar and delivering the pellet subcutaneously between the scapulae greater than 5 mm from the point of skin penetration. Care will be taken to avoid contact of the pellet with any liquids prior to insertion under the skin, as this may alter the drug delivery characteristics of the pellet. No further intervention is necessary to close the skin.

Table 1: Treatment Groups: Group Numbers and Dose Codes

Nominal Dose for a 30-g mouse (mg/kg/day)	Isradipine Dose	Color Code	Group
0	0 µg/day by continuous delivery from s.c. pellet (placebo); 90-days drug per pellet	Yellow	1
5	150 µg/day by continuous delivery from s.c. pellet; 90-days drug per pellet	Blue	2

6.0 EVALUATION OF EXPERIMENTAL SUBJECTS

6.1 In-Life

- *Pre-dose baseline*: body weights, and detailed functional observations, including neurological signs (See [Attachment A](#)).
- *Once daily (morning)*: clinical observations.
- *Once daily (afternoon)*: cage-side checks
- *Weekly*: body weights, detailed functional observations including neurological signs (See [Attachment A](#)).

6.2 Necropsy (see also [Attachment B](#))

- *Unscheduled deaths*: Perform a general necropsy and record any gross pathology findings. Record the location, weight and appearance of the implanted pellet, including, by visual inspection, the approximate percentage of pellet remaining. In general, tissue will not be saved from unscheduled deaths due to possible artifacts from autolysis.
- *Moribund (see clinical criteria, Attachment C) or terminal (scheduled) necropsy*:
 - *Neuropathology* (~half of the mice in each treatment group): Anesthetize with pentobarbital (~50 mg/kg, i.p.). [Note: second injection s.c. if necessary to maintain plane of anesthesia, up to 85 mg/kg total dose; may adjust initial dose within a range of 40-85 mg/kg, i.p. to optimize anesthesia]. Flush blood via intracardial perfusion with phosphate buffered saline (PBS) followed by 4%

paraformaldehyde. Remove brain and dissect (forebrain and brainstem), and dissect a spinal cord segment. Using pre-labeled sample containers, store tissue in fixative at 4°C until overnight shipment on wet ice. Record the location, weight and appearance of the implanted pellet and, by visual inspection, the approximate percentage of pellet remaining.

- *Biochemical analysis* (~half of the mice in each treatment group): Terminate under CO₂. Immediately remove and dissect brain into olfactory bulb (OB), cortex/hippocampus (CTx/H), midbrain (MB), cerebellum (Cblm), and brainstem (BrS), and spinal cord (SC). Freeze samples in pre-labeled cryovials on liquid nitrogen or dry ice and store at -80 °C until overnight shipment on dry ice. Then complete a gross necropsy and record gross pathology findings and fix gross lesions in 10% NBF for optional future histopathology. Record the location, weight and appearance of the implanted pellet and, by visual inspection, the approximate percentage of pellet remaining.
- *Tissue Shipments*: Neural tissues will be shipped on a pre-arranged schedule by overnight delivery to M K Lee (or designee) at the University of Minnesota (contact and shipping address to be provided). (Note: the targeted number of animals per treatment group for neural tissues may represent a mixture of males and females).

6.3 Post-Mortem Endpoints (University of Minnesota)

- Anti-bodies for alpha-synuclein and ubiquitin (qualitative histology and optical density of stained sagittal brain sections; and western blot for brain homogenates).
- Axonal degeneration (sagittal sections, brainstem) using silver degeneration stain (qualitative histology and optical density).
- Astroglial activation (sagittal sections, brainstem) using glial fibrillary acidic protein (GFAP).

7.0 DATA COLLECTION AND ANALYSIS

7.1 Data Collection

Body weights, dose volume, and clinical observations at dosing may be recorded in Provantis[®] Instem[™] or recorded on paper using study-specific data collection sheets. Detailed functional observations, including neurological signs will be recorded on paper using a study-specific data collection sheet.

7.2 Statistical Comparisons

The comparisons of interest are the main effects of treatment (i.e., vehicle vs. isradipine), sex (male vs. female), and age (week of age). The number of animals on study is expected to decrease progressively through the duration of the study. Statistical tests will be selected by the designated statistician based on characteristics of the data sets for specific endpoints (e.g., Kaplan Meier survival analysis; parametric or nonparametric statistical analyses as appropriate to the endpoint; multi-factorial ANOVA; trend tests and pair-wise comparisons, as applicable).

7.3 Interpretation

The following findings would be considered evidence of potential therapeutic activity:

- Delay in onset and/or reduction in severity and/or delayed progression of neurological symptoms (i.e., clinical observations at dosing and/or detailed functional observations, including neurological signs) and/or extension of life span.
- Amelioration of post-mortem findings (incidence and/or severity) as interpreted by collaborator, M.K. Lee, Ph.D., University of Minnesota.

9.0 LITERATURE CITATIONS

9.1 In-Life

Lee, MK et al. (2002). Human α -synuclein-harboring familial Parkinson's disease-linked Ala-53 \rightarrow Thr mutation causes neurodegenerative disease with α -synuclein aggregation in transgenic mice. *PNAS*, 99 (13), June 25, 2002.

NRC (1996). National Research Council. Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC.

Smith, WW et al. (2010). Synphilin-1 attenuates neuronal degeneration in the A53T α -synuclein transgenic mouse model. *Human Molecular Genetics (HMG Advanced Access)* March 20, 2010).

9.2 Statistics

Estimating Kaplan-Meier Survival Curves:

Kaplan, EL and Meier, P (1958), "Nonparametric Estimation from Incomplete Observations," *Journal of the American Statistical Association* **53**, 457-481.

Proportional Hazards Regression Models:

Cox, DR (1972), "Regression Models and Life Tables (with discussion)," *Journal of the Royal Statistical Society Series B*, **34**, 187-220.

Logrank Test for comparing survival distributions:

Mantel, N. (1963). Chi-square tests with one degree of freedom: extensions of the Mantel-Haenszel procedure. *J. Amer. Statist. Assoc.*, **58**, 690-700.

Mantel, N. (1966). Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother. Rep.*, **50**, 163-170.

Peto, R. and Peto, J. (1972). Asymptotically efficient rank invariant test procedures (with discussion). *J. Roy. Statist. Soc. Ser. A*, **135**, 185-206.

Tarone, R. and Ware, J. (1977). On distribution-free tests for equality of survival distributions. *Biometrika*, **64**, 156-160.

ATTACHMENT A

Detailed Functional Observations
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Evaluations will be conducted at pre-dose baseline and weekly thereafter. Endpoints were selected from a routine functional observational battery (SOP LST-NBT-005) and specific neurological signs were derived from published and unpublished observations of the phenotype. Findings will be recorded on a customized data collection form for this study. Mice may be excluded from functional observations if clinical condition precludes collection of meaningful data. Reason(s) for exclusion will be documented in the study record.

Functional Observations:

- *Muscle Tone*
- *Forelimb Placing*
- *Approach*
- *Startle*

Neurological Signs (2-min in open field, note signs including but not limited to the following):

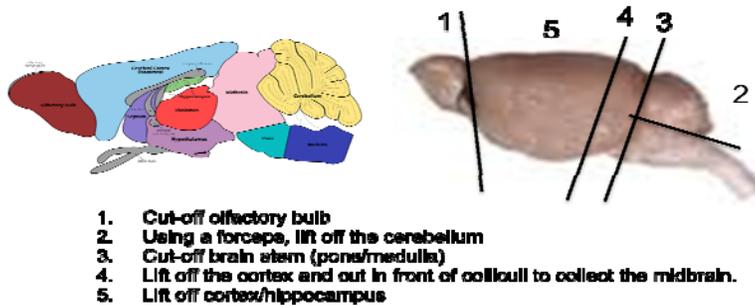
- *Tremors*
- *Convulsions*
- *Retropulsion* (walking backwards)
- *Sustained posturing* (particularly unusual posture; give brief description)
- *Abnormal limb position* (limb held up; specify which limb)
- *Abnormal limb position* (limb dragging; specify which limb)
- *Limping* (specify which limb)
- *Episodic running* (sudden, repeated bursts of running and stopping)
- *Bradykinesia* (slowness of movement; mild, moderate, severe)
- *Akinesia* (inability to initiate movement; briefly describe circumstances and duration)
- *Rigidity* (increased muscle tone with resistance to passive movement; mild, moderate, severe)
- *Postural instability – Standing* (difficulty maintaining upright position; mild, moderate, severe)
- *Postural instability – Rearing* (difficulty rearing or maintaining rearing posture, may fall over attempting to rear; mild, moderate, severe)
- *Ataxia* (mobile but with gross lack of coordination for muscle movements, e.g., staggering; mild, moderate, severe)
- *Dystonia* (sustained muscle contractions causing twisting and repetitive movements or abnormal postures; mild, moderate, severe)
- *Manipulation of Nestlets* (normal = fully shredded and mixed with bedding; grade 1 = <70% shredded ($\geq 30\%$ intact); grade 2 = <30% shredded ($\geq 70\%$ intact); grade 3 = not shredded, essentially intact). Standardize the number of days between adding a Nestlet to the cage and scoring for manipulation of Nestlets. The date on which Nestlets are added to the cage, as well as the date of observations will be maintained in the study records.

ATTACHMENT B

Supplement for Collection and Processing of CNS Tissue

Decapitation. When removing the head, use hand-held instruments (do not use a guillotine as this may damage the spinal cord).

Fresh Frozen Tissue for Biochemical Analysis. Olfactory bulb (OB), Cortex/Hippocampus (CTx/H), Midbrain(MB), Cerebellum(Cblm), Brain Stem(BrS), and Spinal Cord. Take precautions to preserve structural integrity at the time of dissection and freezing.



Dissect out the vertebral column from the cervical through lumbar vertebrae. Use gauze to hold the vertebral column section. Place the tip of a 5-ml syringe filled with saline directly over the opening of the vertebral column at the lumbar end of the cross section. Flush the intact spinal cord out of the cervical end of the vertebral column. Place the spinal cord in a cryotube and quick freeze on liquid nitrogen or dry ice. Store in ultra cold freezer at -80°C . Ship overnight on dry ice.

Intracardial Perfusion:

- Deeply anaesthetize the mice and flush the blood with phosphate buffered saline (PBS) at ambient temperature (~ 50 ml at ~ 5 - 10 ml per min via gravity perfusion).
- Change the perfusion solution to 4% paraformaldehyde (PFA) in PBS at ambient temperature (~ 100 ml at ~ 5 - 10 ml per min via gravity perfusion).
- Decapitate and carefully remove the brain with brain stem and post-fix in 4% PFA/PBS for 24-48 hrs, use at least 25-40 ml per brain. Store in PBS/0.02% sodium azide solution at 4°C .
- Remove the spinal column (with the bones). Place in 4% paraformaldehyde (PFA)/PBS for 48-72 hrs. Store in PBS/0.02% sodium azide solution at 4°C .

Fixative: Intracardial perfusion is preferred to assure the best quality tissue. If perfusion cannot be performed, use 4% PFA in PBS (fresh within the week and 0.45 μm filtered). Remove fresh brain, bisect on the mid-sagittal plane, and immerse in fixative (~ 40 ml per brain) for 72 hrs (with one change). Store in PBS/0.02% sodium azide solution at 4°C .