Dye tracing protocol; Dianna Kahn 10/03

To obtain tissue:

Embryos: remove from mother, chill in PBS and decapitate. Let head bleed out in PBS for a few minutes. Place head into 4% para in 4 degrees at least overnight before removing brain from the skull.

Pups and adults: Euthanize pups (P0-P3) with hypothermia, Older pups and adults with lethal injection IP. If using chlorohydrate use 0.5 cc for pups and 1 cc for adults (of a 5% solution in PBS). Once respiration has ceased, peruse first with 0.9% saline (this step is not necessary for P0-P3), then with 4% para made in 0.1M PB or 1Xpbs, filtered and Ph'ed to 7.4. After perfusing, remove brain from skull and post-fix over night in 4% para, at 4 degrees. NOTE: you do not need to make 4% para fresh each time. You can either make a 10% solution in water, and keep it at 4 degrees for up to a month, or make the 4% para, filtered and Ph'ed, then aliquot and freeze at –20.

Dye injection:
After at least overnight 4 degree post-fixation (up to a couple months), two types of fluorescent carbocyanide dyes, DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) and DiA (4-4-dihexadecyl aminostyryl N-methyl-pyridinium iodide; Molecular Probes) can be used. You may first hemisect the cortex along the mid-saggital sinus if you wish to do another type of experiment in the opposite hemisphere. For dye placement, first take a digital image of the hemisphere and superimpose a pre-constructed grid on the image to determine dye placement location. This grid is used for reliability of injection site location across cases, although an experienced eye works well too. Once the position for dye insertion is determined, make a small hole in the pia at the injection site location, using a 36 gauge syringe needle-tip, and insert the DiI or DiA crystal into the cortical tissue, through the superficial and middle layers of the cortex. It is better to use insect pins with handles as a tool for insertion. If you use these tools, you will re-use the pins, so make sure you only use one dye color per tool. Care should be taken to avoid penetration of the crystal into

the underlying white matter. The hemisphere should be digitally imaged a second time in order to record exact definition of dye placement.

Tissue storage and processing:

The injected hemisphere should be stored in darkness at room temperature for 6-8 weeks, sometimes longer, in 4% paraformaldehyde to allow for transport of the tracers. If you are using hemisected brains, you can have a look at it before cutting, under the FL scope, to see whether dye has reached the diencephalons; this is a way to check if the transport is complete. After the dye transport period, the hemisphere should be lightly dried with a kim wipe, and embedded in 5% low melting-point agarose. Once you pour the agarose over the brain (inside the mold) make sure to stir it around to remove any excess para from the brain. Section tissue into 100μm sections (60 is the minimum), in the coronal or saggittal plane, using a vibratome. You cannot freeze dye brains! All sections should be immediately counterstained for Hoechst (Aldrich Chemicals) to visualize nuclear and laminar boundaries, then mounted in phosphate buffer onto glass slides and coverslipped with Vector.

NOTE: FL photos should be taken immediately, as the dye 'leaks' out of the sections very quickly, even if they are coverslipped.

NOTE: to make DAPI (Hoechst) 2 microliter Hoechst in 10 ML 1X PBS.