ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

TECHNICAL WHITE PAPER:
ALLEN DEVELOPING MOUSE BRAIN ATLAS

ATLAS OVERVIEW

The Allen Developing Mouse Brain Atlas provides a characterization of gene expression in the brain beginning with mid-gestation through to aging. Building upon the foundation established by the original Allen Mouse Brain Atlas, the Allen Developing Mouse Brain Atlas provides a framework to explore temporal and spatial regulation of gene expression, effectively a 4D atlas, with a highly accessible and easily navigable free online database designed to be used in conjunction with expert-annotated reference atlases also provided on the website.

This public resource, available via the Allen Brain Atlas portal (www.brain-map.org), is designed in order to meet the following objectives: 1) Profile genes functionally relevant to brain development or developmental disorders of the brain; 2) Visualize the development of cell types and brain regions using robust neuroanatomical markers; 3) Offer a platform to allow the user to access and understand the dynamics of gene expression through development; and 4) Provide easy search and navigation of the dataset to enable discovery of new genes with important roles in brain development.

Data sets include:

- ~2000 genes characterized by *in situ* hybridization (ISH) in sagittal plane across four embryonic and three early postnatal ages, as well as a subset of genes surveyed in aged brain.
- ~60 developmental marker genes characterized by ISH in coronal plane of section across the same embryonic and early postnatal ages.
- ~20 developmental marker genes characterized by ISH in coronal plane with higher temporal resolution, including 8 embryonic timepoints and 6 early postnatal timepoints.

PIPELINE OVERVIEW

The Allen Developing Mouse Brain Atlas utilizes the production processes as developed for the Allen Mouse Brain Atlas, a genome scale atlas of gene expression in the mouse brain (Lein, Hawrylycz et al. 2007), with adaptations including: 1) addition of a yellow counterstain to enhance analysis of the ISH; 2) changes in tissue embedding processes for embryonic tissue; 3) adjusted proteinase K concentrations optimized for each age; 4) adjusted Nissl protocols for some timepoints; and 5) utilization of dual image acquisition platforms.

Gene Selection

This genelist can be broken down into the following categories:

1. *Transcription factors.* Approximately one third of the genes are transcription factors, with extensive coverage of homeobox, basic helix-loop-helix, forkhead, nuclear receptor, high mobility group and POU domain genes.
2. *Neuropeptides, neurotransmitters, and their receptors.* Extensive coverage of genes in dopaminergic, serotonergic, glutamatergic, and gabaergic signaling, as well as neuropeptides and their receptors. This category of genes was also surveyed in aging brain.
3. *Neuroanatomical marker genes*. Characterizing region- or cell-type specific marker genes over development can provide information about the origins of a brain region or cell type, and may help to identify precursor regions at earlier timepoints.

4. *Gene ontologies/signaling pathways relevant to brain development.* Gene ontologies include axon guidance, receptor tyrosine kinases and their ligands. Pathways include Wnt Signaling and Notch Signaling pathways.
5. *Genes of general interest.* This category includes highly studied genes such as common drug targets, ion channels, cell adhesion, genes involved in neurotransmission, G-protein-coupled receptors, or involved in neurodevelopmental diseases, which are expressed in brain in the adult and/or in development.

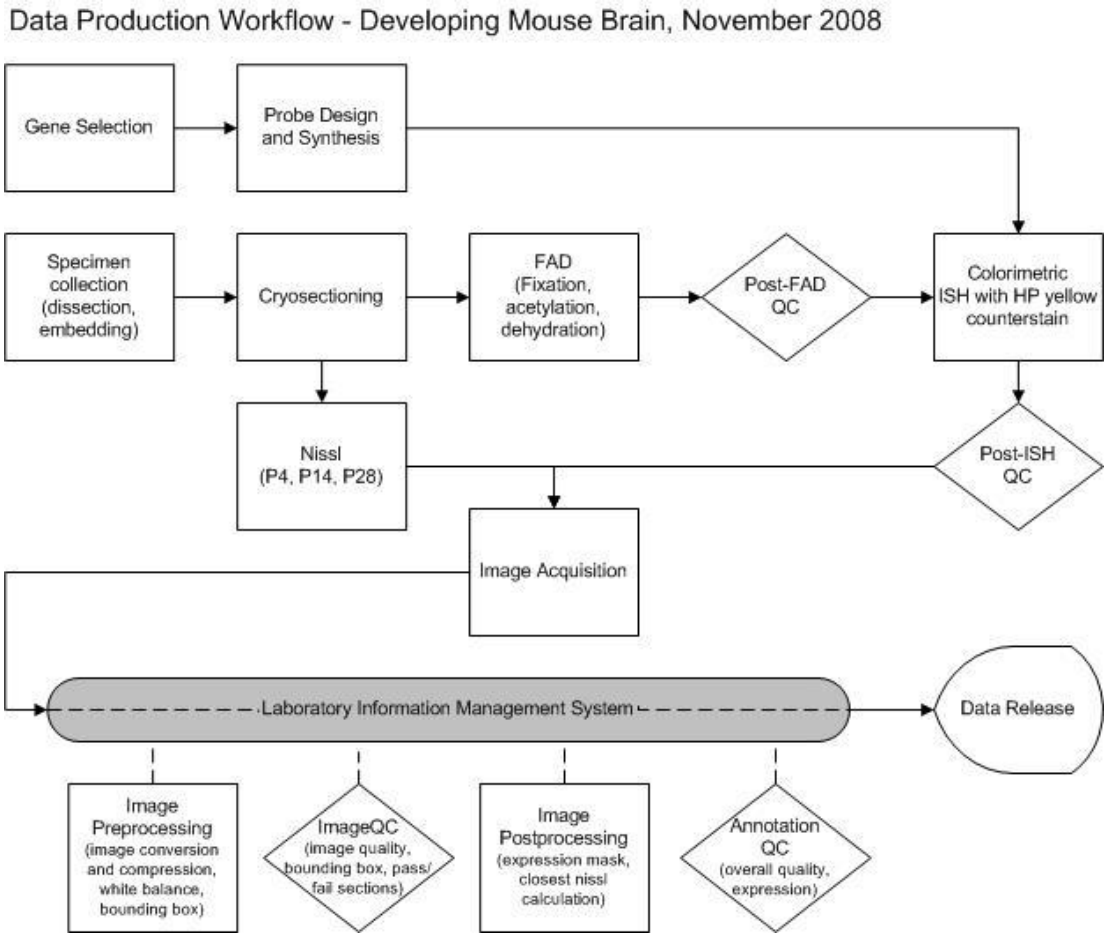

Figure 1. Workflow for Data Production for the Developing Mouse Brain as of November 2008.

Probe Design and Synthesis

The procedures described in the Allen Mouse Brain Atlas Data Production Processes were used for generating probes for the developing mouse brain atlas. The three sources of probe templates are cDNA clones, pooled cDNA from mouse brain, and synthetic cDNA clones.

cDNA clones. When cDNA clones are available from the MGC (Mammalian Gene Collection, NIH), they are used as direct templates for PCR. The clones are stored as glycerol stock in 384-well and 96-well plates at -80 °C. Clone sequences are verified by comparison to RefSeq sequences. Consensus sequences with >98% homology across 80% of the total length are used to develop probes.

cDNA templates. When clones are unavailable for a given gene, pooled cDNA reactions made from mouse brain total RNA is used as a template source. Probes are generated against sequences within 3000 bp from the 3' end of the cDNA.

Synthetic cDNA clones. In rare instances splice variants make cDNA template design impossible, so we order synthetic plasmids from GenScript (www.genscript.com). We pull sequence information from NCBI's RefSeq database. We submit this sequence information to GenScript. GenScript then synthesizes the 400-500bp clone insert. We then use the resulting synthetic plasmid as the direct template for PCR amplification.

Mouse brain cDNA preparation. Total RNA is isolated from homogenized C57BL/6J mouse whole brain tissue using Ambion's ToTALLY RNA kit, or Trizol and Ambion's MagMaxExpress protocol. Typical yield is 120 µg total RNA per brain. Invitrogen's Superscript III RTS First-strand cDNA synthesis kit is used for cDNA reactions in a 96-well format, using 5 µg Anchored olig-dT-25.

Primer design. Gene-specific forward and reverse primers are designed in the following way. BLAST is used to identify regions of homology in other genes/family members, and repetitive and/or homologous sequences are masked. Primer3 software is used for primer design with the following criteria:

1. Optimal size is 18-20 nt for clone templates, 22-24 nt for cDNA templates
2. GC content between 42-62%
3. PCR product size between 300-1200 nt (optimal > 600)
4. Probe location within the gene (No bias for clone templates, within 3000 bp of polyA tail for cDNA templates)

The top primer pair is chosen with the lowest penalty score. A nested reverse primer is also designed for cDNA templates. The SP6 RNA polymerase binding sequence is added to the reverse/nested primer. Primers are ordered from IDT in 96-well format at 10 µM concentration.

All gene sequences are blasted against available collections of transcript sequences including RefSeq, MGC< Celera, TIGR, Riken, and UniGene. Regions of homology greater than 70% for regions over 100 bp are identified and excluded from probe design. (For a subset of genes in families with high homology these standards were relaxed to >90% homology over 120 bp). Within the remaining sequence, primers were designed using Primer3 software (MIT). A nested approach is used for the generation of probes from mouse brain cDNA, such that three primers are generated: a forward, a reverse, and a nested primer. An initial polymerase chain reaction (PCR) is performed using forward and reverse primers. The purified product is then used as a template for a second PCR using the same forward primer with the nested primer. When a cDNA clone is used as a template, a single PCR reaction is used with a single set of forward and reverse primers. All PCR products generated from cDNA templates are sequenced from both ends, using the forward primer and SP6.

In vitro Transcription (IVT). Standard IVT reactions are performed using Roche's 10X DIG RNA Labeling Mix. All reactions are done in 96-well format for 2 hours at 37°C, with 30 µl total volume. Purified PCR products serve as the template, using SP6 RNA polymerase (NEB). IVT reactions are purified using Millipore's Montage 96 filter plate, and are eluted with 90 µl of THE (0.1 mM Sodium citrate pH 6.4, Ambion) following a 30 minute room temperature incubation. IVT reactions are quantified using RiboGreen HIGH assay (Molecular Probes) and the SpectraMax-M2 plate reader (1.0 µl in 200 µl total volume). 1.0 µl of each IVT reaction is analyzed on Agilent's Bioanalyzer 2100 for size confirmation and quantification. IVT reactions are stored at -80°C.

Quality Control. PCR products are evaluated for expected size and homogeneity; PCR products with multiple products are discarded. IVT products that are shorter than their predicted size are also discarded; however, it is common to see IVT products slightly larger than their predicted molecular weights, or to see multiple peaks, due to RNA secondary structure. IVT products with multiple bands are not used for ISH unless the additional bands are determined to result from secondary structure.

Dilutions. IVT reactions are diluted to working stocks of 30 ng/µl with THE. For hybridization, probes are diluted to 300 ng/ml into hybridization buffer (Ambion) in 96 well plates, and are stored at -20°C until use.

Specimen Preparation

The C57BL/6J mouse strain was chosen for the developing mouse brain atlas because the strain is widely used in the research community, and to permit direct comparison to both the Allen Mouse Brain Atlas (adult P56 mouse brain) and to the Allen Spinal Cord Atlas (P4 and P56 mouse spinal cord). Seven timepoints were chosen for broad characterization of gene expression, including four embryonic timepoints and three postnatal timepoints. The embryonic timepoints were chosen to span a wide range of developmental events in utero, beginning with embryonic day (E) E11.5 and ending with postnatal day (P) P28.

Breeding. Specimens were derived from breeding pairs for all timepoints except for P28. Breeding animals were either purchased from The Jackson Laboratory—West or were derived from purchased animals. Personnel monitored the presence of vaginal plugs at 12 hour intervals (6 am and 6 pm). In order to harvest embryonic specimens with accuracy to 0.5 days, only dams with visible plugs at 6 am were used to obtain embryonic timepoints. For postnatal timepoints, births were recorded at 12 hour intervals (6 am and 6 pm). Animal handling was reduced as much as possible for P4 and P14, and animals were maintained on a 12 hour light-dark schedule. The P28 animals were either bred in house and weaned at day 21, or obtained from The Jackson Laboratory, with shipping at P21, receipt of animals at P23, and maintained under normal housing conditions for 5 days prior to dissection.

Aged animals. For aging studies of between 18 months (18M) and 24 months of age (24M), mice were selected from The Jackson Laboratory C57BL/6J colony at 10 weeks of age. They were housed 5-10 per cage and aged at The Jackson Laboratory until 17 months of age. Mice were then shipped to the Allen Institute and aged an additional month for 18M timepoint or 5 months for the 24M timepoint. These mice were housed in the same groupings as at Jackson Laboratory, but no more than 5 per cage. Environmental enrichment was provided for all animals. Early pilot work was performed using a small subset of brains obtained from 30-33 month mice from a colony at University of Washington, and early pilot work on 24M animals was performed on mice aged entirely at the Allen Institute.

Specimen Criteria. In order to obtain the most homogenous set of specimens for each timepoint, certain criteria were established (see Table 1). For each embryonic timepoint, specific Theiler stages (TS; (Theiler 1989) were chosen for production, representing the most prevalent Theiler stage occurring at that age, as determined using major criteria from Karl Theiler. For E11.5 and E13.5 embryos, gender was not determined. Male specimens were used for E15.5 through adult. For P14 collection, all pups within the harvested litter displayed eye opening. Specific specimen criteria were not established for brains obtained for E12.5, E14.5, E16.5, E17.5, P1, P2, and P7, except for selection of gender (male).

Table 1. Specimen Criteria

Age	TS	Major criteria	Gender (method)
E11.5	TS19	Clear anterior footplate; No sign of fingers; Pigment not uniformly distributed around eye	Undetermined
E13.5	TS21	Anterior footplate indented; Posterior footplate beginning to indent; fingers not yet separated distally; Pinna forms crest pointing 90 degrees out of head	Undetermined
E15.5	TS24	Eyelids closing; Anterior part of the back is straight; Nail primordial on toes; Head is rounded not angular	Male (tail-tip, genotype)
E18.5	TS26	Long whiskers; Pinna fused to skin; Males are showing a lump and dark pigment between the anus and genitals	Male (visual)
P4	N/A	Body weight > 1.8 grams	Male (visual)
P14	N/A	Body weight > 6 grams; Eyes open	Male (visual)
P28	N/A	Body weight > 10 grams	Male (visual)
≥18M	N/A	Exclude for health issues during visual inspection and necropsy (skin lesions, abscesses, abnormal organs)	Male (visual)

Specimen embedding. Whole embryos aged E11.5 through E15.5 were dissected into individual wells of a 12-well plate containing chilled 4% sucrose/PBS for 15 min. During the sucrose equilibration, embryos were analyzed under a dissecting scope to determine Theiler stages for individual

embryos. Embryos meeting the Theiler stage criteria were then stepped through 50:50 OCT:4% Sucrose/PBS for 2 min prior to freezing in OCT in a dry ice alcohol bath. For E16.5 and E17.5 specimens, whole heads were removed and degloved, then equilibrated and embedded in the same manner as the embryos. E18.5 and postnatal specimens were examined for basic specimen criteria, and the brain was dissected and frozen directly in OCT. Brains are frozen at -80 ºC prior to sectioning.

Cryosectioning

Specimens were sectioned at 20-25 um (See Table 2) on a Leica 3050 S cryostat with adjacent sections placed across different slides to produce series of slides such that when a given series was used for ISH or Nissl it would result in the sampling shown in Table 2. Nissl series were generated for every specimen at ages P4, P14, and P28, and these associated Nissl images are available on the website.

Table 2a. Sectioning schemes for major timepoints

Ages	Specimen type	Section width	Genes per brain	Sampling per gene	Nissl sampling
E11.5	embryo	20 µm	4	80 µm*	N/A
			* sagittal only; coronal is 120 µm		
E13.5	embryo	20 µm	5	100 µm	N/A
E15.5	embryo	20 µm	6	120 µm	N/A
E18.5	brain	20 µm	7	140 µm	N/A
P4	brain	20 µm	7	160 µm	160 µm
P14	brain	25 µm	7	200 µm	200 µm
P28	brain	25 µm	7	200 µm	200 µm
P56	brain	25 µm	6	200 µm	100 µm
18M	brain	25 µm	6	200 µm	100 µm
24M	brain	25 µm	6	200 µm	100 µm

Table 2b. Sectioning schemes for additional timepoints

Ages		Section width	Genes per brain	Sampling per gene	Nissl sampling
E12.5	embryo	20 µm	4	80 µm	N/A
E14.5	embryo	20 µm	5	100 µm	N/A
E16.5	head	20 µm	6	120 µm	N/A
E17.5	head	20 µm	7	140 µm	N/A
P1	brain	20 µm	6	140 µm	140 µm
P2	brain	20 µm	7	160 µm	160 µm
P7	brain	20 µm	8	180 µm	180 µm

Fixation, Dehydration, Acetylation

Fixation, dehydration, and acetylation (F/A/D) were performed as described for the Allen Mouse Brain Atlas (Data Production Processes). For all ages except E14.5 and E16.5-P7, sections were allowed to air dry on slides for 30 minutes prior to F/A/D. For E14.5, and E16.5 – P7 tissue, the tissue was first air-dried and then baked overnight in a 37 °C oven prior to F/A/D, in order to improve tissue adhesion to the slide. The tissue is then fixed in 4% neutral buffered paraformaldehyde (PFA) for 20 minutes and rinsed for 3 minutes in 1x PBS. Acetylation is performed to reduce non-specific probe binding to tissue sections. The tissue is equilibrated briefly in 0.1 M triethanolamine and acetylated for 10 minutes in 0.1 M triethanolamine with 0.25% acetic anhydride. Immediately following acetylation, the tissue is dehydrated through a graded series containing 50%, 70%, 95%, and 100% ethanol. Finally, each slide is analyzed microscopically to ensure section quality. Slides that pass QC are stored at room temperature in Parafilm-sealed slide boxes for up to one month.

***In situ* Hybridization**

In situ hybridization (ISH) processes are performed as described in the Allen Mouse Brain Atlas (Data Production Processes), using a non-radioactive, digoxigenin (DIG) based technique to label cells expressing a particular mRNA sequence.

The ISH protocol is performed using a Tecan robot with GenePaint technology developed by Dr. Gregor Eichele's Laboratory at the Max Planck Institute and Baylor College of Medicine. The ISH protocol executed on the Tecan platform is detailed in Appendix I. Slides are integrated into flow-through chambers on a temperature-controlled rack, and placed on a Tecan Genesis liquid handling platform. Reagents are applied using a liquid handling system. Prior to hybridization, the fixed, acetylated, and dehydrated tissues undergoes steps designed to block endogenous peroxidase activity and to increase permeability of the tissue, allowing penetration and hybridization of the labeled probe to its complementary target mRNA. The tissue is incubated with digoxigenin-labeled riboprobe for 5.5 hours at 63.5 °C. Once hybridization is complete, the tissue is treated with a sequence of increasingly stringent washes containing decreasing salt concentrations.

Detection of the bound probe is a multi-step procedure. First, a succession of blocking steps inhibits endogenous protein activity from interfering with the colorimetric enzymatic reactions. The colorimetric reaction itself is a four-part process, starting with addition of a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody. A tyramide signal amplification (TSA) step is utilized to maximize sensitivity. The tissue is incubated with a biotin-coupled tyramide. Tyramide is converted to HRP into a highly reactive oxidized intermediate which binds rapidly and covalently to cell-associated proteins a or near the HRP-linked probe, resulting in amplification of bound biotin molecules available for detection by up to a hundred fold. These biotin molecules are then bound to neutravidin-AP. A colorimetric reaction occurs when the alkaline phosphatase (AP) conjugated to neutravidin enzymatically cleaves the phosphate from 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and two of the resulting indoles undergo a redox reaction with nitroblue tetrazolium (NBT) to produce a blue-purple precipitate at the site of probe binding. Once this process is completed, the tissue is treated with a wash buffer containing EDTA followed by fixation with 4% PFA to halt the colorimetric reaction.

The major modification to the original protocol is the optimization of proteinase K concentrations to obtain the highest *in situ* hybridization signal while retaining tissue integrity. The proteinase K concentrations are listed in below (Table 3); postnatal timepoints P14 and P28 are processed under the same conditions as the P56 tissue in the Allen Mouse Brain Atlas.

Table 3. Proteinase K treatment.

Ages	Proteinase K (U/mL)	Time in Proteinase K
E11.5	0.0014 U/mL	1 x 10 min
E12.5-P7	0.0025 U/mL	1 x 10 min
P14-adult	0.0175 U/mL	2 x 10 min

Every ISH run has three control slides on age-matched tissue: 2 positive controls and 1 negative control. Positive control genes were selected by the following criteria: 1) expression at all timepoints examined; 2) expression across many brain regions, such that most brain sections will show evidence of expression; and 3) expression of varying intensity at each timepoint including areas of no expression, low/medium expression, and high expression. Positive controls are Cannabinoid receptor 1 (*Cnr1*) and Calbindin 2 (*Calb2*), as shown in Figure 2.

Yellow Counterstain

The Feulgen-HP yellow DNA counterstain is a nuclear stain that adds definition to the tissue for the purpose of analyzing and understanding the gene expression data. This counterstain is used in conjunction with ISH for all data produced for the Allen Developing Mouse Brain Atlas, except for P56, in order to provide tissue context to the ISH signal which is otherwise difficult to discern due to the very light tissue background for embryonic ISH. The counterstain also enables better tissue detection and focus during automated image acquisition.

After colorimetric ISH is completed on the tecan robots (Data Production Processes), the slides are removed and undergo an acid alcohol wash (70% ethanol adjusted to pH 2.1) to reduce background, 5N hydrochloric acid washes to prepare the tissue for HP yellow counterstain, followed by HP yellow counterstain (Catalog #869, Anatech Ltd) and two final acid alcohol washes to remove non-covalently bound HP yellow. Slides are coverslipped using Hydro-Matrix Mounting Medium. The yellow counterstain and acid alcohol washes are carried out using a Leica CV5030 coverslipper. Coverslipped slides are incubated overnight at 37 °C to solidify the mounting media. Prior to scanning, slides are cleaned to remove excess mounting media and other debris.

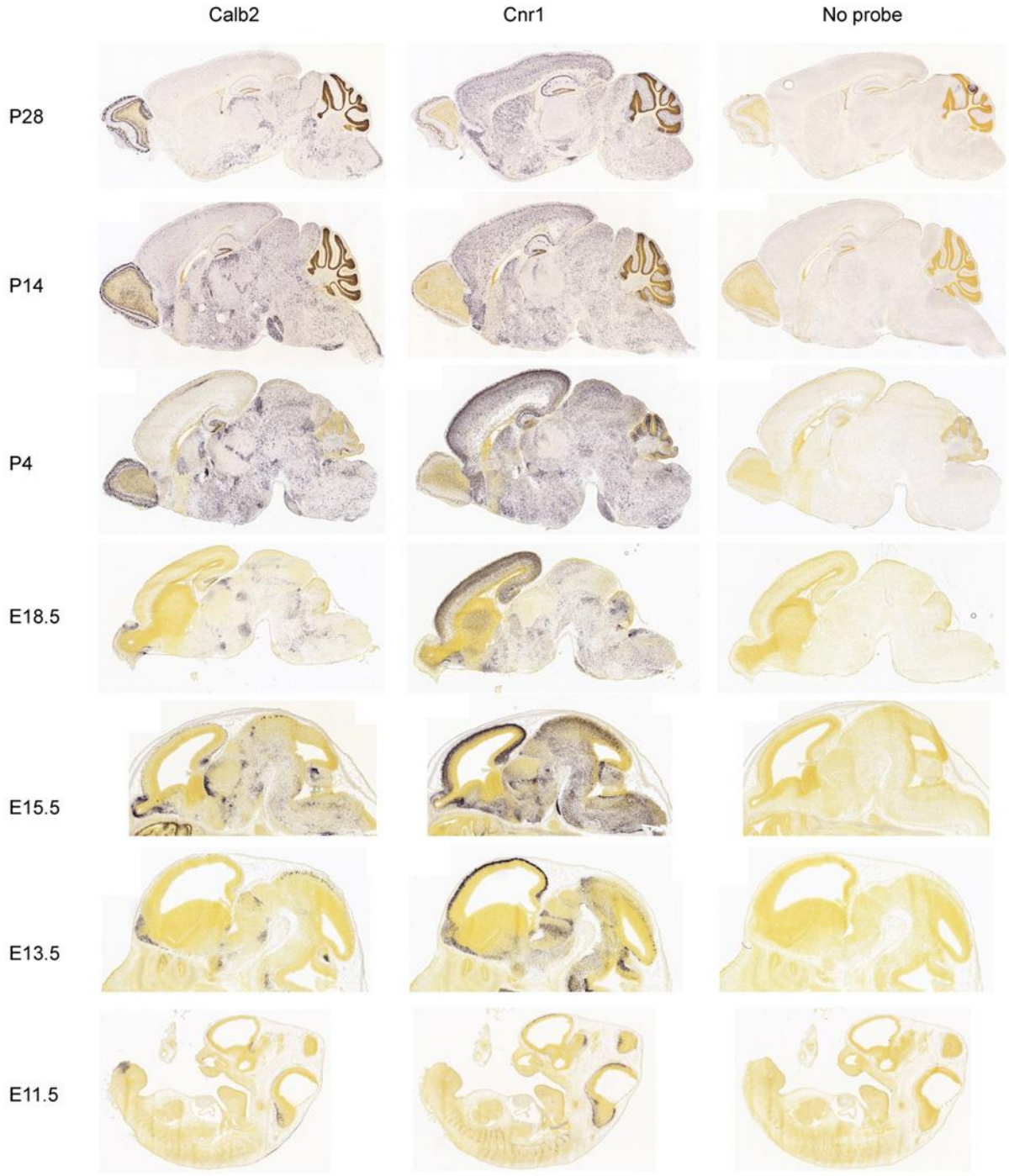

Figure 2. Examples of ISH controls for each timepoint using the probes *Calb2*, *Cnr1*, and a no probe control. Adjacent tissue sections are used for each of the control experiments on each tecan run.

Nissl staining

Nissl staining is a brain-specific histological technique that labels Nissl substance, the ribosomal RNA associated with rough endoplasmic reticulum. In adult and postnatal brains, Nissl staining serves as a cytoarchitectural reference to help identify specific cell populations in the brain; however, at earlier times in brain development, this stain gives no more information than a nuclear stain, such as the Feulgen-HP yellow

counterstain present on all ISH datasets. Nissl sets were generated for all postnatal timepoints at sampling densities indicated in Table 2.

There are a variety of dyes that stain Nissl substance, including thionin and cresyl violet. The nissl protocol using 0.25% thionin stain described in the Allen Mouse Brain Atlas Data Production Processes was used for P14 and P28 tissue. For P1-P7 tissue, the only modification that was made to the protocol was the substitution of 0.72% cresyl violet/60 mM sodium acetate, pH 3.4 for the thionin stain.

Briefly, after sectioning, a set of slides from each P4, P14, P28, P56, 18M, or 24M brains is baked at 37°C for 1-5 days. Sections are defatted with xylene substitute Formula 83 and hydrated through a graded ethanol series (100%, 95%, 70%, and 50% ethanol). After incubation in water, slides are stained in either thionin or cresyl violet, differentiated and dehydrated in water and a graded ethanol series (50%, 70%, 95%, and 100% ethanol). Finally, slides are incubated in Formula 83 and coverslipped in DPX mounting medium. Slides are air-dried in a fume hood at room temperature.

Image Acquisition
Slides for P14, P28, P56, 18M, and 24M tissue were scanned on the same Image Capture System (ICS) platform developed for use for the Allen Mouse Brain Atlas (Data Production Processes). All other timepoints (E11.5 - P7) were scanned using the ScanScope automated slide scanner (Aperio Technologies, Inc; Vista, CA) equipped with a 20x objective and Spectrum software, and whole slide images were downsampled to a resolution of 1.0 µm/pixel.

Data Processing
The automated image processing workflow leverages the Informatics Data Processing pipeline generated for the Allen Mouse Brain Atlas (Refer to the Informatics Data Processing pdf) with specific informatics modules created for this project. Following image acquisition on the ICS platform, individual section images are "stacked" or combined into a single slide image, which then enters the same pipeline as the Aperio images. All slide images are white-balanced, and a tissue detection algorithm assigns bounding boxes to individual tissue sections, which are manually assessed and adjusted when necessary. A segmentation algorithm creates the expression mask, which is provided as a colorized view of expression levels across the tissue. The position of each section in the brain or specimen is calculated to a master section index which provides a framework for section position across all timepoints. For P4, P14, P28, 18M, and 24M, the closest nissl section is calculated for each ISH section.

Quality Control
Quality control measures are implemented throughout the process. There is a quality control step for section quality post-fixation just prior to ISH. Post-ISH quality control consists of examination of both positive and negative control slides as well as a random sampling of experimental slides. After image acquisition, image quality is assessed for focus and bounding box. Finally, the data analysis team ensures that all passed images presented to the public meet an acceptable standard for consistency and are of sufficient overall quality for release. First, each specimen is screened for anatomic anomalies, dissection damage, Theiler stage, midline and orientation. If a specimen does not pass this initial evaluation then the entire specimen and all related image series are failed and rerun. Second, each image series is reviewed for sectioning quality, ISH quality, and scanning artifacts. Slides that contain sections with artifacts that can be mitigated are sent back to an earlier stage to be remedied, after which the image series is reassessed by the data analysis team. Third, any individual sections that are unanalyzable are failed and are not released to the public. When greater than 20 percent of the sections are failed, the entire image series is failed.

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Appendix I – Allen Institute *in situ* hybridization protocol

Day 1

Cycles	Time (min)	Volume (µl)	Reagent	Temp (°C)	Total Time (min)	Process
5	5	300	3% H2O2 in MeOH	25	25	Pre-Hyb
7	5	300	PBS (1)	↓	35	↓
2	5	300	0.2M HCl	↓	10	↓
4	5	300	PBS (2)	↓	20	↓
1	5	400	PK(+) buffer	↓	5	↓
1-2*	10	300	Proteinase K *	↓	10-20*	↓
7	5	300	PBS (3)	↓	35	↓
2	10	300	4% PFA (1)	↓	20	↓
7	5	300	PBS (4)	↓	35	↓
2	15	300	Hyb Solution	↓	30	↓
_	15	_	Temperature ramp-up	63.5	15	↓
1	_	300	Riboprobe addition	↓	_	Hybridization
1	330	_	Hybridization	↓	330	

Day 2

Cycles	Time (min)	Volume (µl)	Reagent	Temp (°C)	Total Time (min)	Process
5	5	300	5 x SSC	61	25	Stringency Wash
5	10	350	Formamide I	↓	50	↓
5	12	350	Formamide II	↓	60	↓
4	8	300	0.1 x SSC (1)	↓	24	↓
1	8	300	0.1 x SSC (2)	25	8	↓
4	5	300	NTE (1)	↓	20	Post Hyb Blocking
3	5	300	20 mM Iodoacetamide (1)	↓	15	↓
3	5	300	20 mM Iodoacetamide (2)	↓	15	↓
4	5	300	NTE (2)	↓	20	↓
2	5	300	TNT (1)	↓	10	↓
3	5	300	4% Sheep serum (1)	↓	15	↓
3	5	300	4% Sheep serum (2)	↓	15	↓
4	5	200	TNT (2)	↓	20	↓
2	10	300	TNB blocking buffer	↓	20	↓
2	5	200	TNT (3)	↓	10	↓
2	5	300	Maleate wash buffer (1)	↓	10	↓
2	10	350	Blocking reagent	↓	20	↓
2	5	300	Maleate wash buffer (2)	↓	10	↓
2	5	250	TNT (4)	↓	10	↓
3	5	350	TMN	↓	15	↓
4	5	200	TNT (5)	↓	20	↓
4	10	300	TNB blocking buffer	↓	40	↓
2	30	350	Anti-DIG-POD	↓	60	Colorimetric Detection
6	5	250	TNT (6)	↓	30	↓
1	30	250	Tyramide-biotin	↓	30	↓
6	5	300	Maleate wash buffer (3)	↓	30	↓
2	20	350	Neutravidin	↓	40	↓
6	5	300	Maleate wash buffer (4)	↓	30	↓
4	5	250	TNT (7)	↓	20	↓
2	5	400	TMN	↓	10	↓
2	15	350	BCIP/NBT	↓	30	↓
1	10	350	BCIP/NBT	↓	10	↓
3	_	400	System liquid (1)	↓	10	↓
1	_	300	NTE (3)	↓	5	↓
1	10	250	4% PFA (2)	↓	10	↓
1	_	400	System liquid (2)	↓	10	↓

* Concentration and time dependent upon tissue (see Table 3)