ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

TECHNICAL WHITE PAPER:
ALLEN DEVELOPING MOUSE BRAIN REFERENCE ATLAS

The Allen Developing Mouse Brain Atlas provides a cellular-resolution map of gene expression in the developing mouse brain from the embryo to the young adult. To provide a novel neuroanatomical framework based on genoarchitectonic data, the Allen Developing Mouse Brain Reference Atlas was created with the expertise of Professor Luis Puelles, MD, PhD (University of Murcia, Spain). Sagittal full-color, high resolution Web-based digital reference atlases have been created for seven stages of mouse brain development (**Figure 1**). These are based upon a systematic developmental ontology which is available in 13 hierarchic levels in the Ontology Legend Browser application, and is presently partially implemented down to Level 08.

Purpose

The Allen Developing Mouse Brain Reference Atlases were designed to:

1) Allow users to directly compare gene expression patterns to an annotated developmental atlas.
2) Provide templates for the creation of 3D computer models of the developing mouse brain.
3) Serve as a neuroanatomical foundation for informatics-based analysis tools.

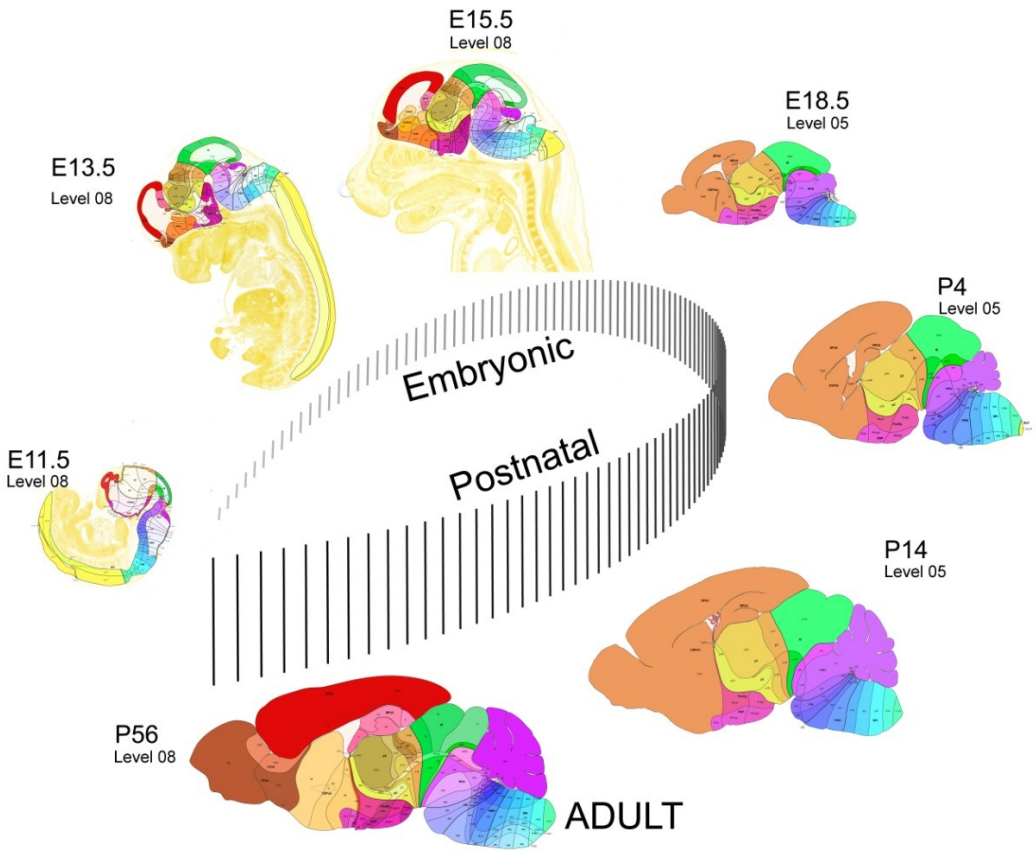


Figure 1. Representative sections of four embryonic and three postnatal mouse brain atlases.

UPDATES FOR MARCH 2010

The March 2010 public release includes the following updates to the atlas and ontology:

- More detailed (updated) ontology.
- Drawings through Level 08 of the ontology for E11.5, E13.5, E15.5, and P56 atlases.
- Updated E18.5 and P4 Level 05 atlases.
- Inclusion of fiber tracts in the ontology and in E13.5, E15.5, and P56 atlases.
- Addition of a second midbrain subdivision (pre-isthmus, or m2).
- Some changes in structure names or abbreviations.
- A revised color scheme to represent the new, more detailed drawings.
- A legend browser application that allows the user to link from the ontology to the reference atlas drawings.

DEVELOPMENTAL ONTOLOGY OF THE MOUSE BRAIN

Overview

At variance with other brain ontologies, which strictly classify adult neuroanatomic entities from a conventional topographic viewpoint, the present ontology was designed to be useful for both developing and adult forms of the mouse brain, employing a topological ontogenetic viewpoint. This means that the location of brain structures is not referred to fixed external references, such as a baseline, a supporting plane, or a stereotaxic frame, and is resolved instead by recourse to constant internal reference landmarks in the irregularly growing brain primordium (e.g., structural details of the brain midline, nerves, and fiber tracts) and unchanging neighborhood relationships of the distinguished parts, supported by a number of well-characterized gene expression patterns. This approach is also supported by data gathered via descriptive embryology and experimental or transgenic fate mapping studies.

Each stage or “level” of the ontology is a topological one-to-many transform of the previous stage, as normally occurs during development, with progressive regionalization (Levels 01-08), stratification (Levels 09, 10) and, eventually, definition of characteristic areas and nuclei (Levels 11, 12). This means that the few early boundaries are permanent and can be followed into their more or less deformed adult positions and shapes. The early parts can be recognized in their intermediate and adult forms across development. New boundaries are gradually added to the picture when they are generated as a result of ongoing patterning and differentiation processes. The possibilities for anatomical subdivision increase proportionately, and are similarly projected to the adult counterparts. Partitioning always proceeds by subdivision, such that no parts are lost, and every novel neighborhood has a name. Such an ontology subdivides the topological space of the neural tube wall with increasing level of detail as development proceeds.

For practical reasons, the actual temporal sequence of developmental events required simplification and rough systematization when represented as a series of levels of ontological classification (e.g., events of different types which partly overlap temporally were separated for convenience into successive stages, and heterochronic events of the same category that occur at slightly different times depending on the brain region, are presented at the same ontological level, as if they occurred simultaneously). Therefore, this ontology is characterized as “developmental” because the concepts underpinning the classification framework (the levels of the ontology) basically reflect a simplified sequence of regionalization events known to occur in the mammalian brain in general, and in the mouse brain in particular. The associated topological approach is crucial for easy extrapolation from one developmental stage to another and, importantly, will serve to link corresponding data of different mammalian or non-mammalian brains.

Ontological Levels

The first stage, corresponding to **Level 00** of the ontology, corresponds developmentally to the unpatterned neural plate, which is the neuroepithelial undifferentiated primordium of the entire brain. When applied to the adult brain, this level of classification refers to characters that are ubiquitous throughout the brain (i.e., that appear in all derivatives of the neural plate). Late neural plate stages and early neural tube stages register

first incipient and then more advanced primary anteroposterior patterning events, which leads to the use of Levels 01-03 of the ontology (see **Figure 2** for schematic of Levels 01-04).

At **Level 01** the early protosegments or tagmata are delimited (i.e., identifying forebrain, midbrain, hindbrain and spinal cord). **Level 02** enters some secondary divisions within those entities, notably in the forebrain and hindbrain. In the forebrain, this means distinction of the secondary prosencephalon (collective primordium of the prospective hypothalamus, eye and telencephalon) from the diencephalon proper (collective primordium for the prethalamus, thalamus and pretectum and correlative tegmental parts). There is experimental evidence supporting the causal separation of the secondary prosencephalon from the diencephalon proper. This ontology thus departs from the older tradition of considering the hypothalamus as a ventral part of the diencephalon, a thesis that lacks causal experimental support so far. The midbrain is not subdivided at this level of the ontology. In the hindbrain, Level 02 allows us to distinguish one from another prepontine, pontine, pontomedullary and medullary compartments, responding to a neuroanatomical need already well established by tradition (even when not expressed in these exact terms). Note that here the ontology purposefully avoids using the classic metencephalon/myelencephalon or pons/medulla categories, judged to be too simplistic and therefore unwieldy relative to the neuromeric subdivisions that need to be considered next. The main regional divisions of the spinal cord should be separated at Level 02, following the schema of Watson and Sidhu (2009), which contemplates prebrachial, brachial, interramal, crural, postcrural and caudal tagmatic morphofunctional units (these spinal subdivisions are not yet implemented in the atlases or in the ontology at the present stage).

Level 03 introduces generally the respective neuromeric or segmental anteroposterior subdivisions. These imply complete transversal parts of the Level 02 entities, where they exist ("complete" meaning that the corresponding boundaries can be traced uninterruptedly from the roof to the floor of the neural tube) (Puelles and Rubenstein, 2003). We introduce two such parts in the secondary prosencephalon (SP), representing caudal and rostral hypothalamus, or CSP and RSP, respectively, each extending dorsally into telencephalic regions (evaginated telencephalon and preoptic telencephalon, or classic telencephalon impar, respectively). The diencephalon proper becomes tripartite (prosomeres 1-3, or p1-p3). These enclose respectively the pretectum, thalamus and prethalamus, as dorsal entities, plus corresponding parts of the underlying tegmentum (see Puelles and Rubenstein, 2003). The midbrain is divided into two segmental regions, representing the classic rostral mesomere 1 (m1) and caudal mesomere (m2) or pre-isthmic domain. Various classical embryologists had pointed out the existence of a thin (scarcely growing) m2 component of the midbrain in several vertebrates (e.g., Palmgren, 1921). Later usage tended to leave out this concept, but recent gene mapping data have provided novel support for it, as well as the name "preisthmus" (Hidalgo-Sanchez et al., 2005). From E13.5 onwards, the m2 domain can be distinctly visualized as a complete neuromere – from roof to floor – by means of its strong *otx2* in situ labeling, which selectively remains in m2 after *Otx2* signal decreases in the inferior colliculus area of the midbrain (see Atlas). On the other hand, the prepontine hindbrain divides into isthmus and rhombomeres 1 and 2, the pontine hindbrain into rhombomeres 3 and 4, the pontomedullary hindbrain into rhombomeres 5 and 6, and the medullary hindbrain into cryptorhombomeres 7-11 (cryptorhombomeres are non-overt or non-morphologically-identifiable segmental units, which nevertheless are demonstrable by their differential molecular identities – e.g., *Hox* gene codes - and singular histogenetic fates; Marin et al., 2008 and unpublished mouse data). There are thus in all 12 transverse parts of the hindbrain at Level 03. At least the isthmus and rhombomere 1 are known to participate in the formation of the cerebellum, whereas the evidence on a postulated rhombomere 2 contribution is still inconclusive. The spinal cord tagmata (not distinguished yet in the current atlas) each subdivide at Level 03 into the characteristic set of mouse spinal cord segments or myelomeres (Watson and Sidhu, 2009). At present, only the first myelomere (my1) is indicated as an example in the ontology, since all myelomeres largely have the same internal structure, but once spinal tagmata (Level 02) and segments (Level 03) are deineated, these subdivisions would appear as repeating units down the spinal cord.

The basic dorsoventral regions are introduced at **Levels 04 and 05**. At Level 04 there appears the fundamental alar-basal boundary along the whole brain, as well as the more dorsal, parallel boundary separating the telencephalon from the hypothalamus (we trace the latter so that the preoptic region falls within the telencephalon, in support of which option there are sound molecular patterning reasons). At Level 05 (see **Figure 3**) we distinguish in addition the roof and floor plates, thus completing the widely used set of four basic longitudinal zones of the brain (His, 1893). This arrangement of dorsoventral zones in two levels allows us to

use concepts such as “pretectum”, “thalamus”, “prethalamus” or “telencephalon”, which in common usage refer to both alar and roof neural wall domains, as well as concepts such as “midbrain tegmentum”, for

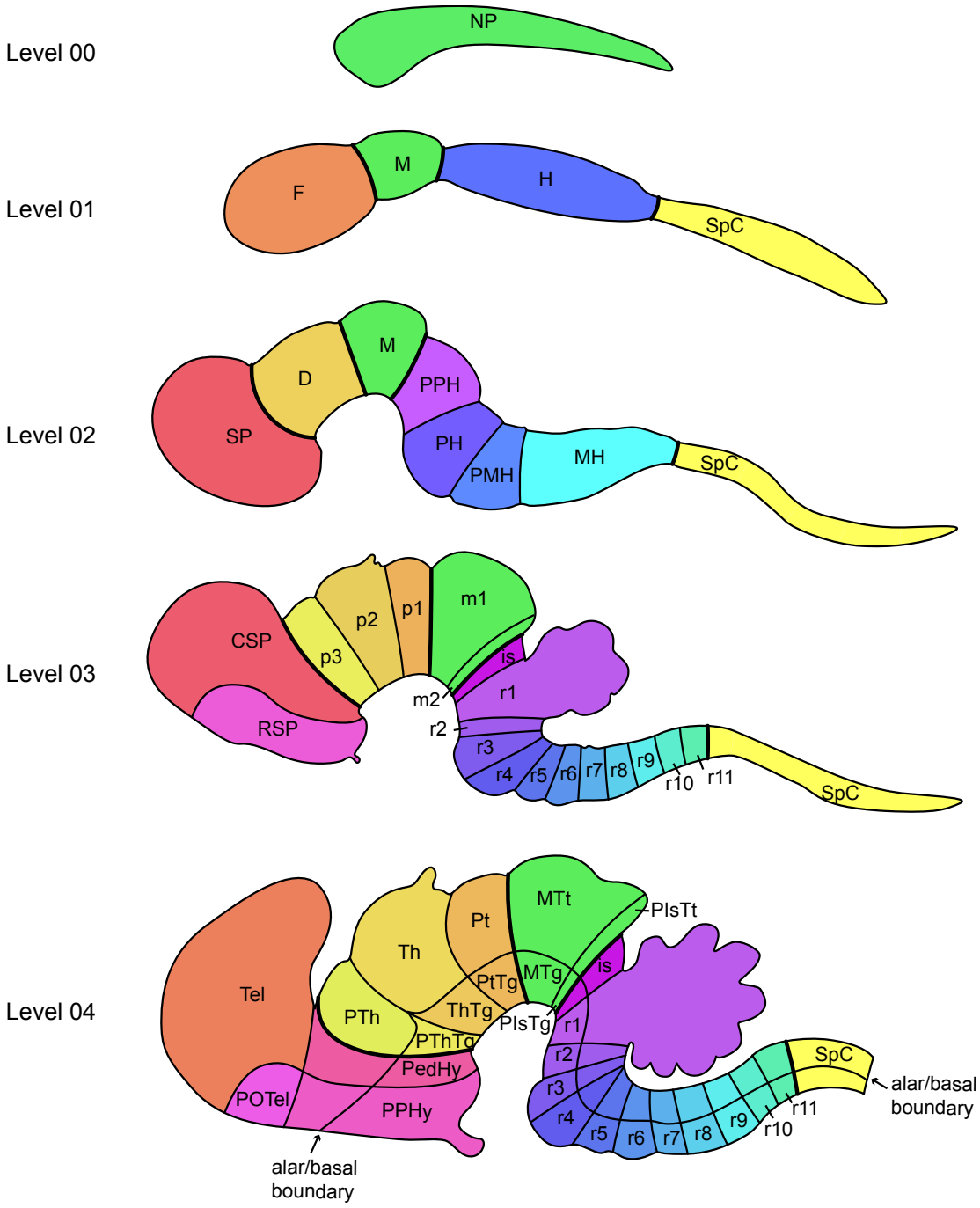


Figure 2. The developmental ontology from Level 00 to Level 04. Beginning with the neural plate (NP) at Level 00, additional levels of neuroanatomical subdivisions are added corresponding to the gradually increasing complexity of the brain through development. Level 01 defines as early protosegments forebrain (F), midbrain (M), hindbrain (H) and spinal cord (SC), with secondary subdivisions appearing at Level 02. By Level 03, neuromeric or segmental anteroposterior subdivisions are depicted. At Level 04, the alar/basal boundary and the telencephalo-hypothalamic boundary are shown. Full names for the acronyms can be found in Figure 4. Schematic drawn by Luis Puelles.

instance, which refer commonly to both basal and floor domains. At Level 04 these entities are unitary, but become separated into the respective dorsoventral parts at Level 05.

Levels 06-08 successively introduce various types of topological subdivision, which are necessary mainly within the telencephalon, though we have also a partial use of them in other brain parts. The high level of regionalization occurring within the enlarged telencephalon forces first recognition of pallium versus subpallium domains (Level 06), then distinct sectors within these parts (e.g., medial, dorsal, lateral and ventral parts of pallium, or striatal, pallidal, diagonal and preoptic parts of subpallium; Level 07), and, finally, finer conventional regions within these units that can be distinguished along the septo-amygdaloid axis, e.g., such as the ”striatal” nucleus accumbens relative to central striatum and the likewise “striatal” central amygdaloid nucleus, or particular septal or claustroamygdaloid subregions (Level 08). These intermediate subdivision levels define the map of known distinct progenitor domains in the telencephalic neuroepithelium. The “diagonal” subpallial domain found intercalated between the pallidal and preoptic domains corresponds in essence to the territory otherwise known in recent literature as “anterior entopeduncular area” (AEP). That term was first introduced in the Bulfone et al. (1993) publication, and has proven to be unintelligible and confusing, since it seems to refer to a population interstitial to the peduncle, but was originally meant to name a complete radial territory extending from a part of the complex of the stria terminalis near the ventricle, across the substantia innominata and basal magnocellular nucleus, into a superficial component coextensive with the diagonal band nuclei. The peduncle, medial forebrain bundle, and internal capsule obviously pass through this domain, which is placed orthogonally to their fibers, along the oblique septoamygdaloid axis. We propose here to change the name of this territory to “diagonal complex” (Dg), whose overall position is easier to visualize by its explicit reference to the clearcut diagonal band nuclei seen at its surface. The Dg is flattened between the lateral preoptic area and the globus pallidus and ventral pallidum.

The Level 06-08 subdivisions are also used in other brain territories where characteristic parts of the alar plate can be distinguished (diencephalon, midbrain, hindbrain), though normally we need less than three levels for this purpose (e.g., to separate superior and inferior colliculi in the midbrain, or nuclear complexes in the thalamus, prethalamus, and pretectum). The pattern is characteristic for each Level 05 alar unit considered, though some common trends may be glimpsed. Up to Level 08, all ontology partitions are conceived to be planar; that is, we have disregarded the third dimension (the thickness of the neural wall), and simplified it to the structure of the undifferentiated pseudostratified neuroepithelium. At the present status of knowledge, these Level 08 planar subdivisions of the brain wall seem to be reasonably complete, although future additional partitioning cannot be excluded. Further regionalization is attributed in the ontology to development of the radial complexity of the cerebral wall in each of the Level 08 areas.

Levels 09-10 attend to basic aspects of this increase in radial complexity, by distinguishing first at Level 09 the primary ventricular and mantle zones of the diverse Level 08 areas. This allows the atlas user to map genes restricted to the ventricular zone cells, and one can also characterize early developmental expression patterns found outside the ventricular zone before definite strata and/or nuclei can be identified. Level 10 advances one step further by defining (somewhat arbitrarily, depending on the locus) periventricular, intermediate and superficial strata of the mantle zone. Of course, the transition of amorphous strata into distinct nuclei, areas, cortical plates and reticular domains occurs heterochronically in different parts of the brain, but each local phenomenon can be straightforwardly attributed to correspond to either Level 09 or Level 10.

Eventually, we approach adult structure with the gradual appearance of the definitive structural complexes of the mature brain. In the ontology, we found that **Levels 11-13** were necessary to classify the anatomic diversity represented in standard atlases of the adult mouse (or rat) brains. For instance, the paraventricular hypothalamic nucleus will appear at Level 11, and its various distinct parts at Level 12. If separate subdomains of the subnuclei need to be delineated by cell typology or chemoarchitectonic criteria, these would be contained within Level 13. In the current version of the ontology there was only a limited need for subdivisions in Level 13.

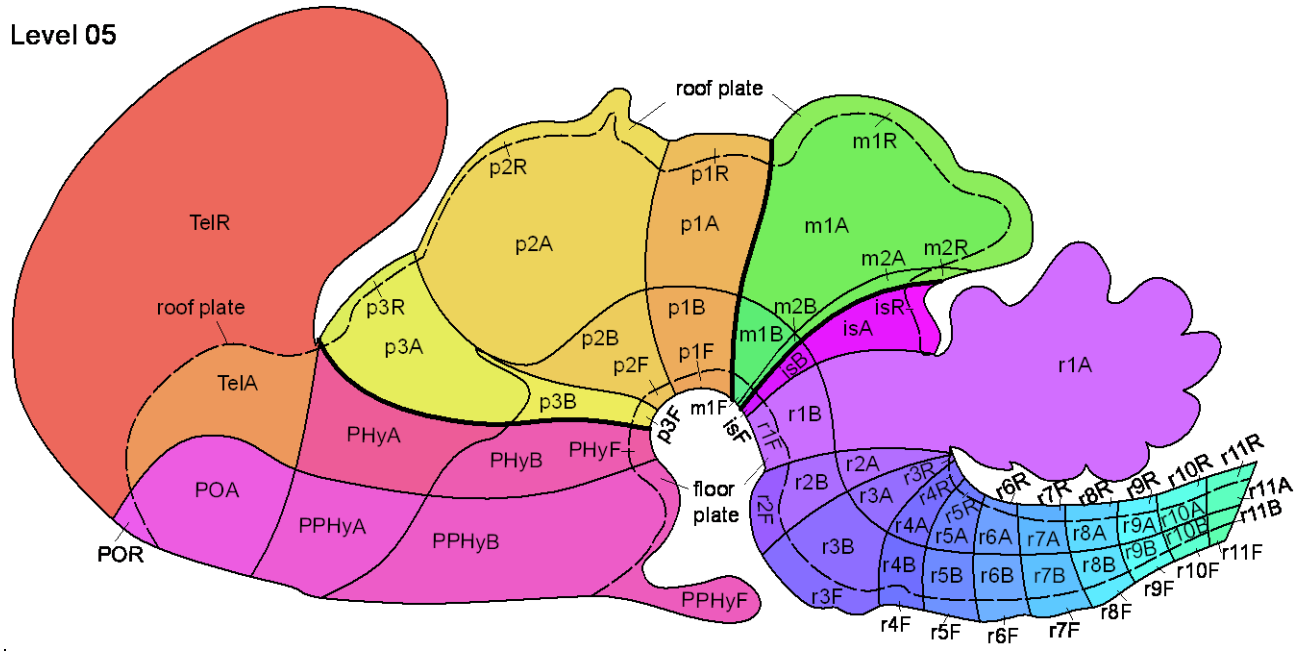


Figure 3. At Level 05 of the ontology, roof and floor plates are delimited.

The final stage contemplated in Levels 11-13 corresponds to the adult, as represented, for instance, in “The Mouse Brain in Stereotaxic Coordinates” (Franklin and Paxinos, 2008). This ontology followed their nomenclature and abbreviation rules for the most part, though some modifications and additions based on other sources, or on novelties implicit in our advanced planar system of subdivisions, were thought to be convenient. At the present time, neither the ontology nor the atlases deal with tangential neuronal or glial migrations. Axonal tracts, decussations and commissures are included in the ontology (listed at the end) and are drawn in several of the atlases (E13.5, E15.5, and P56).

ONTOLOGY LEGEND BROWSER

The reference atlas ontology can be explored by using the Ontology Legend Browser, accessible from the Detailed View window of the Reference Atlases through the (key) icon, or directly from a link to the legend available from the Reference Atlas page. The Ontology Legend Browser application allows the user to expand or collapse different levels of the ontology. An atlas for any given age may be viewed simultaneously with the ontology, and when a structure in the ontology is selected, the atlas image will show a red bounding box indicating the location of the selected structure in the atlas image.

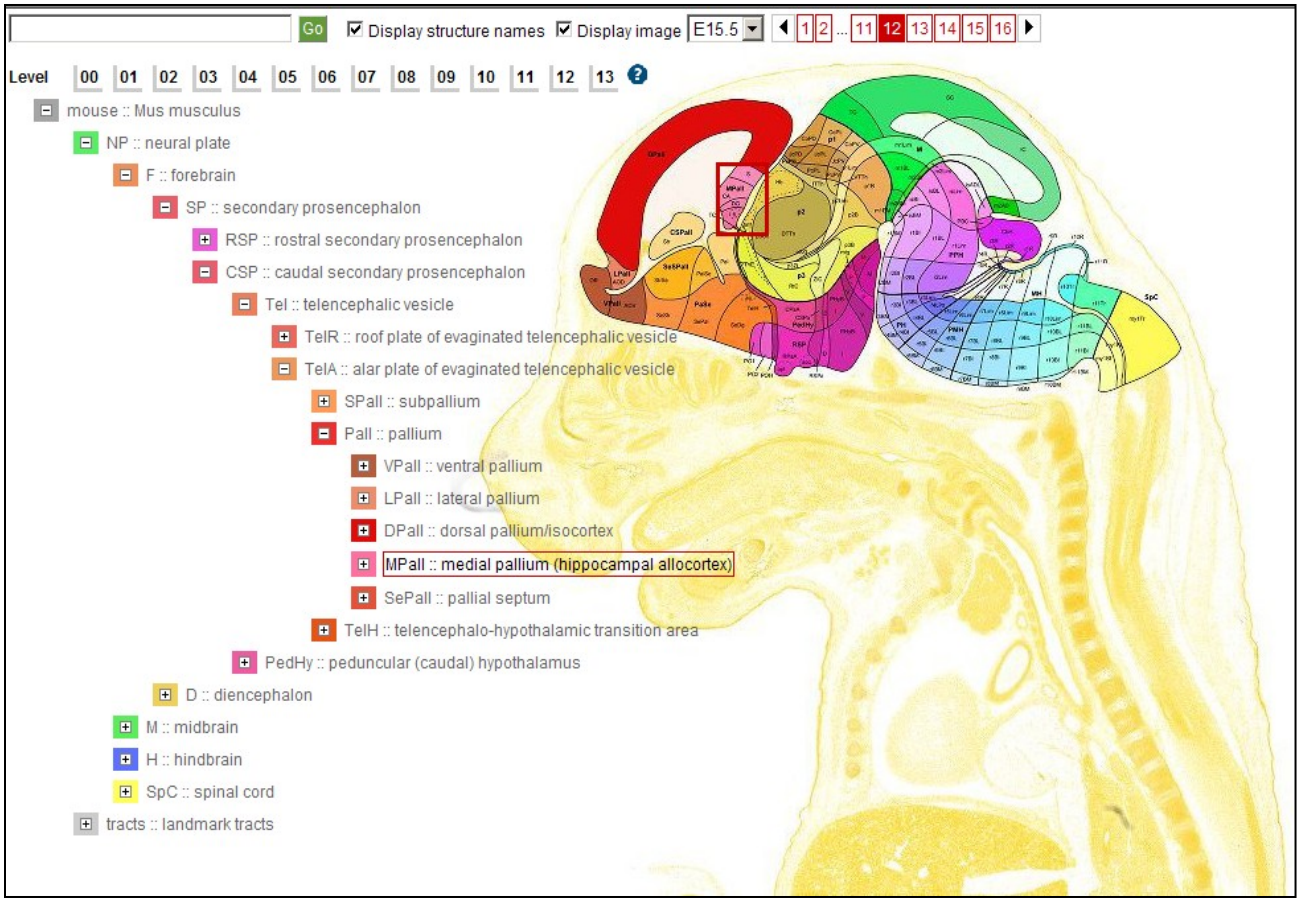


Figure 4. Reference atlas ontology legend browser. The color-coded reference atlas structures are shown organized by hierarchy of the developmental ontology from Levels 00 – 13 alongside a preview image of the reference atlas. The atlas age can be selected at the top.

REFERENCE ATLAS CREATION

Reference Sets

For each atlas, a reference set of images was generated with a histological stain to aid identification of anatomical structures for atlas drawing. **Table 1** provides information regarding the specimens used for atlas annotation. Embryonic (E) specimen age is provided relative to days after conception, with birth expected at approximately 19 days post-conception. Postnatal (P) specimen age is given relative to birth (P0). Theiler stages were determined on the basis of external features identified during dissection and embedding (Theiler, 1989). HP Yellow, a nuclear stain, was used for whole embryo reference sets to allow visualization of all tissues and cells; this stain is also used as a counterstain for the ISH in the Allen Developing Mouse Brain Atlas. Nissl stains were used for all dissected brains to provide additional morphological information of maturing neurons.

Table 1. Details of sagittal reference sets used for atlas annotation.

Age (days)	Theiler stage	Gender	Plane	Stain	Specimen	Section width	Annotated hemisphere	#Annotated Images
E11.5	TS19	N.D.	sagittal	HP Yellow	Whole embryo	20 µm	Right	28
E13.5	TS21	N.D.	sagittal	HP Yellow	Whole embryo	20 µm	Right	15
E15.5	TS24	male	sagittal	HP Yellow	Whole embryo	20 µm	Right	16
E18.5	TS26	male	sagittal	Nissl (cresyl violet)	Dissected brain	20 µm	Left	19
P4	-	male	sagittal	Nissl (cresyl violet)	Dissected brain	20 µm	Left	23
P14	-	male	sagittal	Nissl (thionin)	Dissected brain	25 µm	Left	39
P56	-	male	sagittal	Nissl (thionin)	Dissected brain	25 µm	Left	21

HP Yellow Stain

The Feulgen-HP yellow DNA stain is a nuclear stain that adds definition to the tissue for the purpose of analyzing and understanding the gene expression data. This nuclear stain was used for reference sets created for tissue sections of whole embryo at timepoints E11.5, E13.5, and E15.5. HP yellow is also used as a counterstain 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.

After cryosectioning, the slides are air-dried at room-temperature for 30 minutes, followed by fixation, acetylation, and dehydration (F/A/D) as described in the “Technical White Paper: Allen Developing Mouse Brain Atlas”. Within one month of F/A/D, slides are stained with HP yellow through the following protocol: slides 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 then dehydrated through a graded ethanol series, incubated in Formula 83 (a xylene substitute) and coverslipped in DPX mounting medium. Prior to scanning, slides are cleaned to remove excess mounting media and other debris.

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.

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 P4 tissue, brains were first dissected and equilibrated in 10% sucrose briefly, prior to embedding in OCT. Appropriate Nissl staining for P4 tissue required the substitution of 0.72% cresyl violet/60 mM sodium acetate, pH 3.4 for the thionin stain.

Briefly, after cryosectioning of fresh frozen tissue, a set of slides from each P4, P14, and P28 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.

Annotation of 2D sections

Annotation drawings were done using Adobe Illustrator CS graphics program. The resulting vector graphics were then converted to Scalable Vector Graphics (SVG). Each polygon is then associated with a structure from the ontology: collating polygons in this way allows the flexibility to create various presentation modes (e.g., with or without colorization, transparency and application of different color schemes). The ontology was colorized as shown in **Figure 5** to assist users with identifying structures across different sections and levels.

Archived Reference Atlases

As part of the March 2010 public release, new Level 08 atlases are provided for four timepoints, and minor updates to Level05 atlases are provided for the remaining ages. The original Level05 atlases and the original ontology released as of April 2009 are available as archived pdf documents from the Supplementary Data tab.

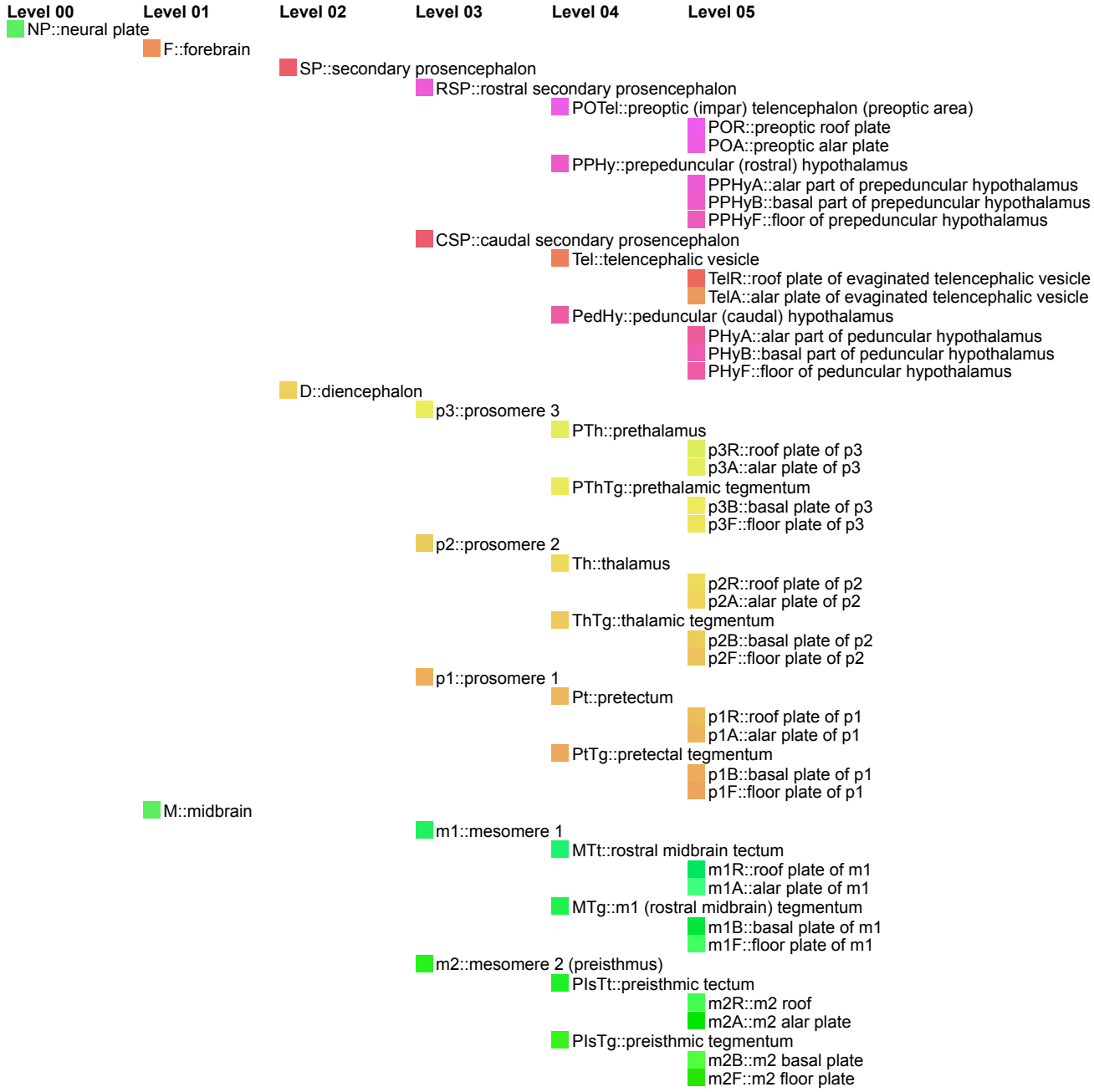
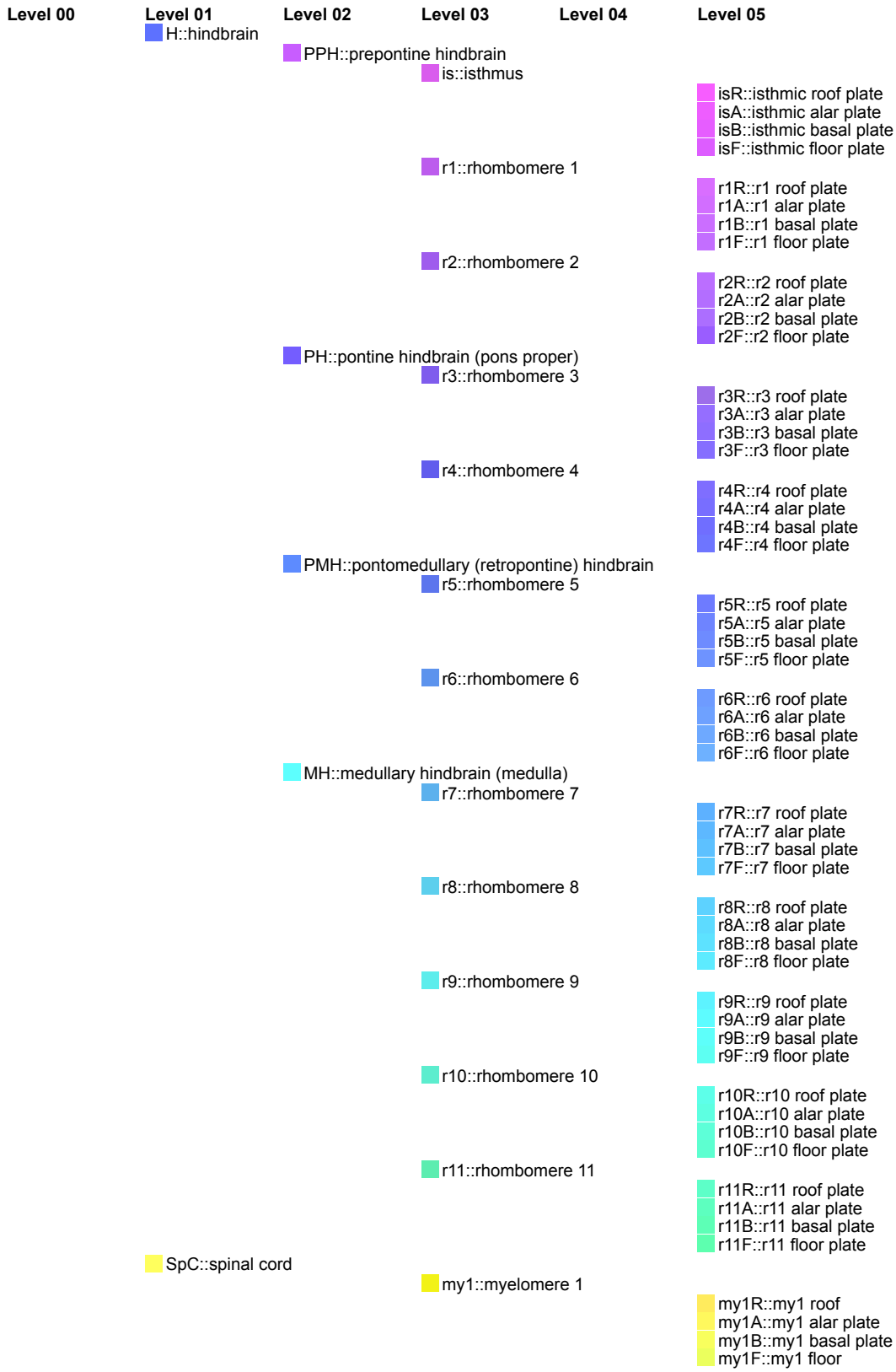


Figure 5. Colorization scheme for the developmental ontology through Level 05. In the presentation of the atlases, brain structures are colorized to allow user to identify the same structure across different sections and different timepoints. The colors were selected such that ontologically related structures are given visually related colors by allocating segments of the color wheel to major subdivisions of the brain: secondary prosencephalon (SP), magenta to orange; diencephalon (D), green/yellow to orange; midbrain (M), green; hindbrain (HB), magenta to blue/green; spinal cord (SpC), yellow. **Continued on next page.**

(Fig. 5, continued)



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