ALLEN INSTITUTE for BRAIN SCIENCE
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ALLEN **Developing Mouse Brain Atlas**

TECHNICAL WHITE PAPER:
REFERENCE ATLASES FOR THE ALLEN DEVELOPING MOUSE BRAIN 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 neuroanatomical framework for expression data, reference atlases for the Allen Developing Mouse Brain Atlas were created with the expertise of Professor Luis Puelles, M.D., Ph.D. (University of Murcia, Spain). Sagittal full-color, high resolution web-based digital reference atlases have been created for seven stages of mouse brain development (**Fig. 1**). A novel anatomic classification system or "ontology" provides ontogenetic relationships between brain regions which is based upon concepts derived from developmental neurobiology, as explained further below.

The reference atlases for the Allen Developing Mouse Brain Atlas were designed to:

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

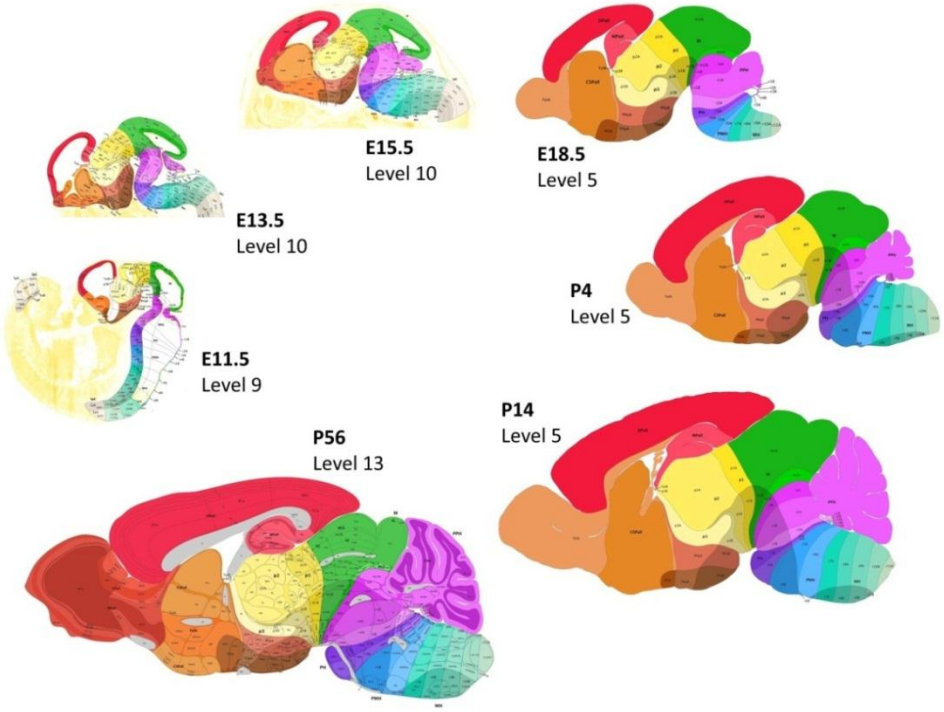

Figure 1. Representative sections of four embryonic and three postnatal mouse brain reference atlases.

DEVELOPMENTAL ONTOLOGY OF THE MOUSE BRAIN

Overview

According to Wikipedia, the philosophical field of ontology deals with questions concerning what entities exist or can be said to exist, and how such entities can be grouped, related within a hierarchy, and subdivided according to similarities and differences. In information science, an "ontology" typically provides a shared vocabulary, which can be used to model a domain, that is, the type of objects and/or concepts distinguished, and their properties and mutual parent-child relations. A brain ontology systematizes hierarchically the parts of the brain as seen from a particular conceptual perspective. 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 to this aim a topological 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.

There are presently 13 levels in our ontology (although additional levels could be added that provide cellular detail such as cell types). 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, stratification and, eventually, definition of characteristic areas and nuclei. 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 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.

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 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 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. 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 are also separated at Level 02, following the schema of Watson and Sidhu (2009), which contemplates prebrachial, brachial, interramal, crural, postcrural and caudal tagmatic morphofunctional units.

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 transverse boundaries can be traced uninterruptedly from the roof to the floor of the neural tube) (Puelles and Rubenstein, 2003). We introduce caudal and rostral neuromeric parts in the secondary prosencephalon (SP), also named recently "peduncular" and "terminal" (prepeduncular) hypothalamus, to avoid the false connotations evoked by the obsolete columnar model widely used before (Puelles et al., 2012). Each of these parts extend dorsally into telencephalic regions (evaginated telencephalon and preoptic telencephalon, or classic telencephalon impar, respectively; **Fig. 2**) representing as wholes the so-called "hypothalamic prosomeres" hp1 and hp2 (ibid). The diencephalon proper becomes tripartite (diencphalic 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, Puelles et al., 2012a,b; Martinez et al., 2012). The midbrain appears divided into two mesomeres (m1, m2; the latter contains the recently identified preisthmic region, intercalated between inferior colliculus and isthmus proper (Puelles, E. et al., 2012)). 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 code - 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. The isthmus and rhombomere 1 participate in the formation of the cerebellum (the vermis being isthmic). The spinal cord tagmata each subdivide into species characteristic sets of spinal cord segments or myelomeres (Watson and Sidhu, 2009); however, individual myelomeres are not represented in this atlas.

The basic dorsoventral regions are introduced at **Levels 04 and 05** (separating for convenience events that coincide in time with AP regionalization covered in levels 1-3). At Level 04, the more dorsal, parallel boundary separating the telencephalon from the hypothalamus appears (we trace the latter so that the preoptic region falls within the telencephalon, for which there are sound molecular patterning reasons), as well as the dorsal and tegmental (ventral) regions associated with the fundamental alar-basal boundary along the whole brain. At Level 05 (**Fig. 3**) we introduce the full complement of roof, alar, basal and floor plates, thus completing the widely used set of 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 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.

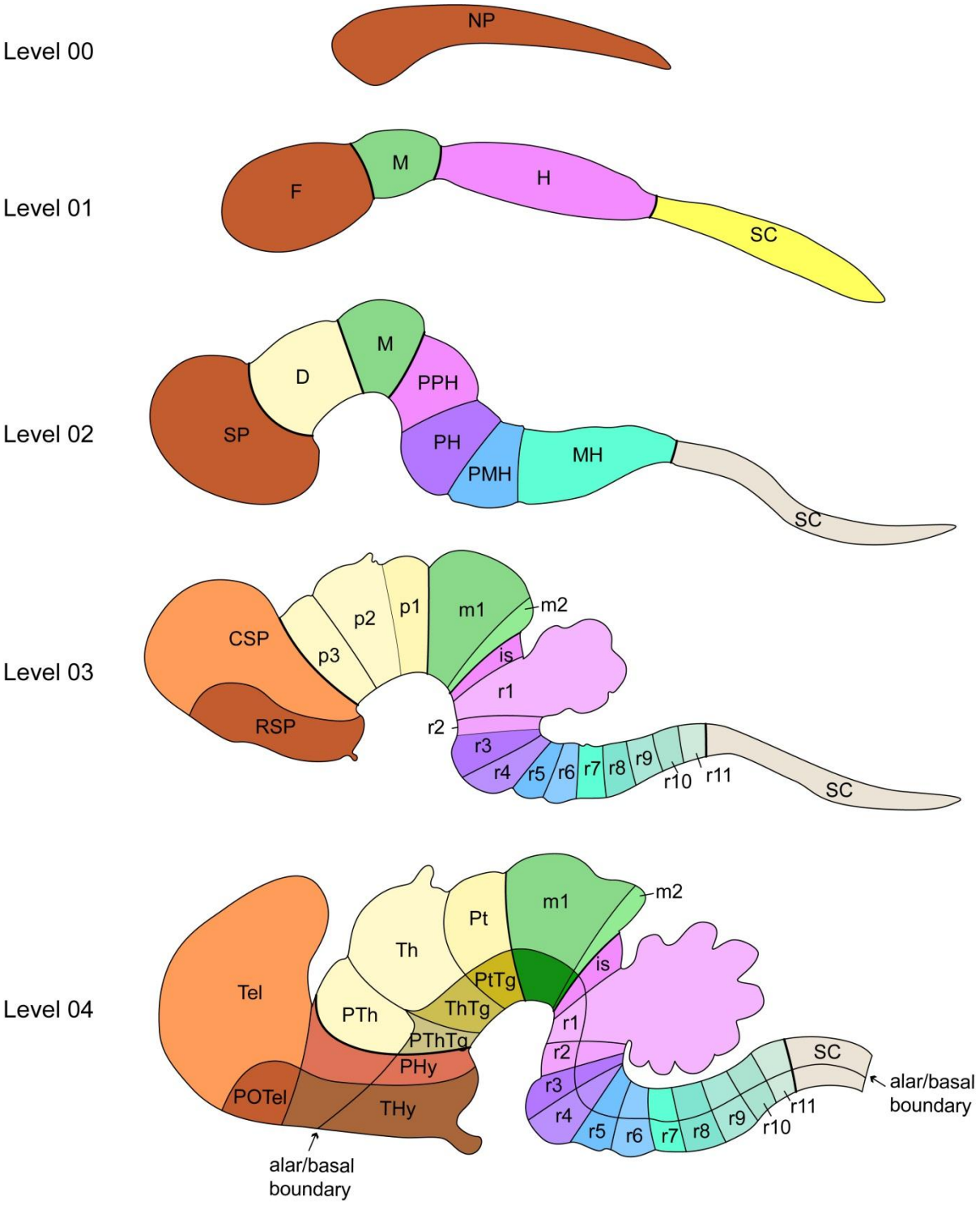

Figure 2. The developmental ontology from Level 00 to Level 04. Beginning with the neural plate (NP) at Level 00, additional levels of neuroanatomical subdivisions are added corresponding to the gradually increasing complexity of the brain through development. Level 01 defines as early protosegments 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 is shown. Full names for the acronyms can be found in Figure 4. Schematic drawn by Luis Puelles.

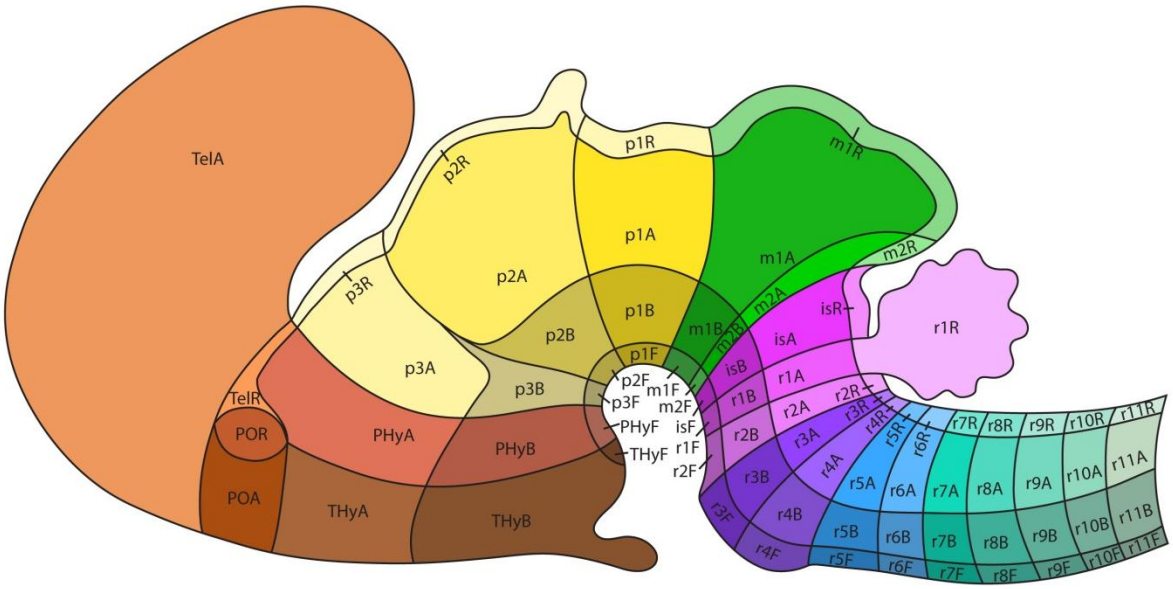

Figure 3. At Level 05 of the ontology, roof and floor plates are delimited.

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 (Level 06), then distinct sectors within these parts (e.g., medial, dorsal, lateral and ventral parts of pallium, or striatal, pallidal, diagonal/innominate and preoptic parts of subpallium; Level 07/08), and, finally, finer conventional regions within these units that can be distinguished along the septo-amygdaloid axis, e.g., such as the area of the nucleus accumbens, or a particular septal or amygdaloid subregion (Level 08). These intermediate subdivision levels are also used in other brain territories where characteristic parts of the alar plate can be distinguished (hypothalamus, 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 or basal 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 so far the third dimension (the thickness of the neural wall is simplified to areal partitions of the pseudostratified neuroepithelium; that is, to the causal origins of structural anatomic blocks in the progenitor domains where given neuronal populations are eventually produced). The planar subdivision of the brain given at this ontology level seems to be essentially complete at the present status of knowledge, so that further regionalization must be attributed to development of the radial complexity of the cerebral wall.

Levels 09-10 attend to basic aspects of this increase in 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, and one can also characterize early developmental expression patterns found outside the ventricular zone before definite strata and/or nuclei can be identified in the mantle. 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 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.

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 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 systematize tangential neuronal migrations, or axonal tracts, due to the difficulties presented by the fact that they traverse different ontology domains (we classify migrated populations at their postmigratory sites, and we merely list the major reference tracts at the end).

Major updates to the ontology in June 2013.
Ontology updates were necessitated by several factors: 1) addition of further detail was required to complete the ontology to level 12/13 for all brain areas (**Fig. 4**); 2) quality control checks conducted during the addition of more detailed atlas items that identified areas of inconsistency between the anatomic reference data and the ontology organization, which were resolved; and 3) conceptual advances in neuroanatomy that needed to be contemplated (e.g., Puelles et al., 2012a,b; Puelles E. et al., 2012; various recent fate-mapping studies).

The major neuroanatomic conceptual advance that influenced alterations to the ontology was a redefinition of the anterior end of the floor plate. In the original reference atlas ontology, the floor plate extended rostrally into the tuberal or infundibular region of the terminal or prepeduncular hypothalamus (encompassing at its end the neurohypophysis and the median eminence). Based upon recent molecular patterning studies (Puelles et al., 2012a) the rostral end of the floor plate has been shifted to the midline between the mammillary bodies (at the terminal or prepeduncular hypothalamus; accordingly the retromammillary and mammillary basal areas lie adjacent to the redefined hypothalamic floor plate; the tuberal midline specializations are ascribed instead to the basal acroterminal midline of the hypothalamus; see Puelles et al., 2012a about this concept).

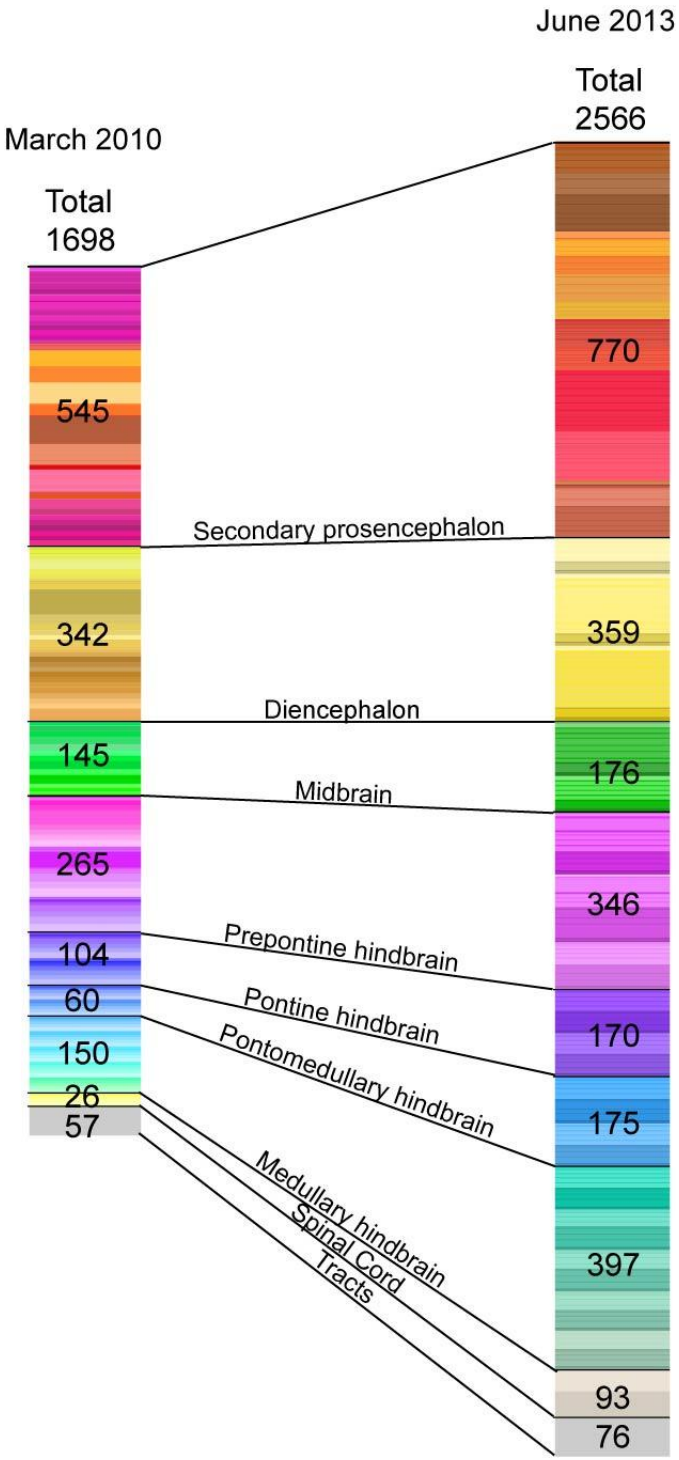

Figure 4. The number of structures in the reference atlas ontology increased from March 2010 to June 2013.
On the left is a color-coded schematic of the number of structures in the ontology from March 2010, and on the right is the corresponding ontology structure from June 2013. Black lines provide the boundaries between major areas of the brain (Level 02 designations). Note the significant increase in detail in the secondary prosencephalon and in the medullary hindbrain. The represented color codes are used correspondingly in the reference atlases (e.g., **Figs.1-3**).

REFERENCE ATLAS CREATION

Reference Sets

For each atlas, a reference set of tissue preparations 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 were 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 a month of F/A/D, slides were 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 were then

dehydrated through a graded ethanol series, cleared in Formula 83 and coverslipped in DPX mounting medium. Prior to scanning, slides were 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. Nissl sets were generated for every P4, P14, and P28 specimen at 160, 200, and 200 microns, respectively.

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, 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, and P28 brains was baked at 37°C for 1-5 days. Sections were defatted with xylene substitute Formula 83 and hydrated through a graded ethanol series (100%, 95%, 70%, and 50% ethanol). After immersion in water, slides were 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 were cleared in Formula 83 and coverslipped in DPX mounting medium. Slides were air-dried in a fume hood at room temperature.

Annotation of 2-D sections

Annotation drawings were done using Adobe Illustrator CS graphics program. The resulting vector graphics were then converted to Scalable Vector Graphics (SVG) and uploaded to the database. Each polygon was then associated with a structure from the ontology. The ontology was colorized as shown in **Figures 1-5** to assist users with identifying structures across different sections and levels. The annotation can be visualized on the online interactive atlas viewer and the annotation SVG are available for download through the Allen Brain Atlas API.

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