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ARTICLE
Novel *in silico* Method for Teaching Cytoarchitecture, Cellular Diversity, and Gene Expression in the Mammalian Brain

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Neuroanatomy can be a challenging topic for undergraduates, making the development of new methods of instruction an important goal of neuroscience educators. In the present report we describe the utility and versatility of the Allen Brain Atlas as a novel tool for instruction of several important anatomical principles of the mammalian central nervous system. Using this digital database, we detail how instructors of laboratory or lecture-based courses can demonstrate cytoarchitecture, cellular diversity, and gene expression profiles of the brain.

Key words: neuroanatomy; neuronal diversity; cytoarchitecture.

A major goal of undergraduate neuroscience education is to provide students with an understanding of the anatomy of the central nervous system (CNS) at both the cellular and systems-levels. Lecture-based courses (in neuroscience, physiology, biology, and psychology) often provide students with two-dimensional photomicrographs and/or illustrations of the CNS similar to those images found in textbooks. In contrast, laboratory-based courses allow students to appreciate the three-dimensionality of the brain with exercises and demonstrations using rodent, sheep, and even human nervous tissue. Together, lecture and lab-based instruction play an important role in the teaching of neuroanatomy to undergraduates.

A number of technical and financial limitations present a significant barrier in the ability of courses to achieve the goal of effectively teaching neuroanatomy. For example, with few (if any) archival tissue specimens, *"How might a laboratory course provide students with representative examples of nervous tissue?"* When archival tissue is available but only few microscopes are present in the lab, *"How might tissue examinations and classroom demonstrations be organized so that all students can participate equally with few students waiting for their turn on the microscope?"* Finally, *"What kinds of laboratory exercises can be implemented requiring minimal laboratory skills on the part of students and without costly reagents (ex. antibodies for immunocytochemistry) or hazardous chemicals (ex. heavy metals)?"*

In silico (ex. web-based) materials offer new, dynamic, and progressive methods for neuroscience instruction and recent reports have documented several web-based exercises and demonstrations for teaching neuroanatomy (Grisham, 2006) as well as neurophysiology (Av-Ron et al., 2006). In this report we provide a novel *in silico* method of teaching the cytoarchitecture, chemoarchitecture, cellular diversity, and gene expression profiles of the rodent brain. We demonstrate the utility and versatility of the Allen Brain Atlas and describe some laboratory demonstrations which will help enhance learning of neuroanatomy.

Description of the Allen Brain Atlas

The Allen Brain Atlas (ABA; www.brain-map.org) is a public database of digital photomicrographs of mouse brains, demonstrating the cellular expression patterns of the mouse transcriptome. As described in the materials and methods sections on the ABA web-site and recently in Lein et al., (2007), brains of male C57BL/6J inbred mice were cut along the sagittal or coronal planes. Using newly-developed methods for automated and high-throughput tissue processing, cryostat sections (25µm) were assayed by *in situ* hybridization (ISH) for >21,000 genes. High-resolution photomicrographs are displayed of the entire rostro-caudal or medio-lateral axis of the brain (coronal and sagittal-cut brains, respectively) separated by ~200µm. A Nissl-stained coronal and sagittal reference atlas is provided and one can navigate through the reference atlases in three dimensions. Colorimetric analysis of every photomicrograph in the ABA is provided, allowing for quantification of gene expression densities. Finally, a number of different search strategies are possible based on gene nomenclature, neuroanatomical region, and expression density.

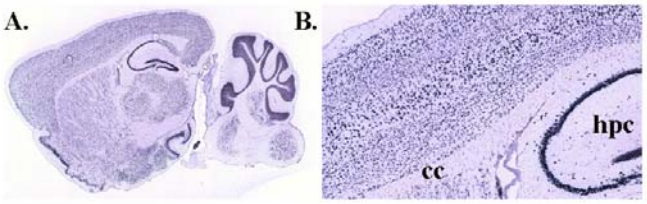

Figure 1. Demonstrating neurons via expression of *Tubulin ß3* (Tubb3) in the mouse brain as revealed in the ABA. Note zoom tool allows for high magnification, revealing individual neurons. Abbreviations: cc, corpus callosum; hpc, hippocampus.

General rationale of teaching strategy using the ABA

Limited histological material or microscopes need not affect the high-quality of instruction necessary to teach neuroanatomy nor limit the "lab experience" for students. Using a video projector and single computer with internet connection, the ABA can be exploited to teach a number of important neuronanatomical principals. Using the ABA, the instructor and students can navigate *together* through the endless resources available in this database. One obvious

drawback is that students are not "touching" specimens or operating microscopes as much as in more traditional courses. However, this is a moot point if these histological and equipment resources are unavailable to begin with. When archival tissue and imaging equipment are available, the exercises and demonstrations described below will surely complement more traditional laboratory exercises.

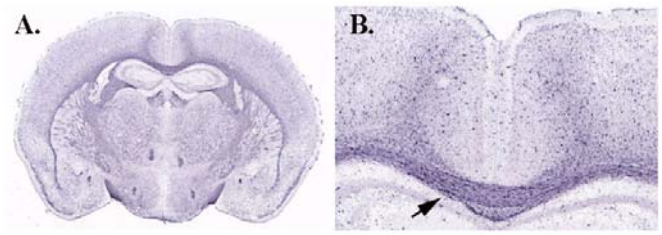

Figure 2. Demonstrating oligodendrocytes via expression of *Myelin basic protein* (Mbp) in the mouse brain as revealed in the ABA. Note robust presence of myelinating oligodendrocytes in corpus callosum (arrow in *B*).

Using the ABA to teach basic cytoarchitecture

The two reference atlases found in the ABA are excellent tools for the instruction of the general cytoarchitecture of the rodent brain. Once the user interface for one of these atlases is open, instructors should remind students of the methodology used in the preparation of the atlas. For example, it is imperative that students understand the rationale for cutting thin sections (25μm) and staining cell somata using the Nissl stain. Next, simply by using the zoom tools, several very important neuroanatomical concepts can be taught effectively. First, the difference between cerebral white matter vs. grey matter can be demonstrated. As Nissl-stained somata in the reference atlas appear blue, students easily appreciate that grey matter areas are comprised of a high density of cells while white matter areas have far fewer cells (discussed below). Next, by zooming in on the neocortex, for example, students appreciate the diversity of cell sizes and their characteristic spatial organization. For example, in neocortex, somata have a characteristic laminar distribution. Finally, exploring subcortical structures like hippocampus and hypothalamus (e.g. paraventricular nucleus), students understand that the spatial organization and packing density of somata create the characteristic appearance of subcortical nuclei. For example, the almond-shaped spatial organization of cells in the amygdala was influential in the naming of this structure. Note that each photomicrograph found in the reference atlases contains a corresponding annotated illustration detailing all cortical and subcortical regions.

Using the ABA to demonstrate cellular diversity in the brain

Undergraduate neuroscience education invariably includes a description of the major cell-types in the brain: neurons and glia. In traditional laboratory classes, students might examine Nissl-stained histological material and identify cells with large (>7 μm) vs. small (<5 μm) somata corresponding to neurons and glia, respectively. Alternatively, students might examine Golgi-stained material demonstrating the full morphology of neurons and glia. In courses with greater resources, students might examine tissue stained with immunohistochemical (IHC) methods against neuron-specific proteins/antigens (ex. neuronal nuclear antigen, microtubule-associated protein 2, β3-Tubulin) and/or glia-specific proteins (ex. glial fibrillary acidic protein, glial high affinity glutamate transporter, myelin basic protein, Ng2-chondroitin sulfate proteoglycan 2). However, obvious drawbacks to conducting IHC exercises in the lab are the high cost of commercial primary and secondary antibodies and the extensive time required (typically more than one day).

The ABA can be exploited to teach the principles of cellular diversity in the brain based on known genetic differences between neurons and glia. Moreover, the different types of neurons (based on transmitter type, projection pattern, etc; described below) and different types of glia (astrocytes, oligodendrocytes, microglia; described below) can be demonstrated. In order to demonstrate neuron-specific expression, instructors and students can navigate through the ABA and examine tissue hybridized for neuron-specific genes. For example, neuron-specific genes include *microtubule-associated protein* genes (Mtap1-9), *neurofilament* genes (Nef3, Nefh, Nefl), and *neuron-specific enolase 2* (Eno2). A list of novel neuron-specific genes identified using the ABA was recently described in Lein et al. (2007). Figure 1 shows the spatial distribution of the neuron-specific gene *Tubulin β3* taken from the ABA. Higher magnification demonstrates the lack of neurons in the corpus callosum (cc), the high density of neurons in hippocampus (hpc), and the laminar organization of neurons in neocortex. Once a representative photomicrograph from the ABA is displayed, students should be instructed to examine the distribution of labeled cells throughout the brain also noting areas devoid of labeled cells. Using this approach students readily appreciate the concepts of grey and white matter, neuronal density differences found throughout the brain, and the spatial distribution of neurons in different brain regions.

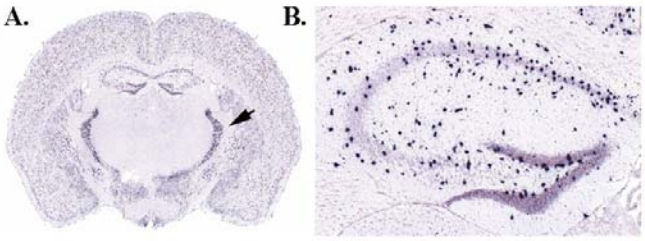

Figure 3. Demonstrating GABAergic neurons via expression of *glutamic acid decarboxylase 1* (Gad1) in the mouse brain as revealed in the ABA. Note robust presence of Gad1 neurons in reticular nucleus of the thalamus (arrow in *A*) and outside the pyramidal cell layer in hippocampus (*B*).

Using the ABA to demonstrate glial-cell diversity in the brain

The distribution of glia and glial-subtypes can be demonstrated using the ABA simply by examining photomicrographs in the database hybridized against glial-

specific genes. The distribution of astrocytes in the brain can be demonstrated by reviewing material in the ABA hybridized for astrocyte-specific genes such as the *Glial fibrillary acidic protein* gene (Gfap). Similarly, oligodendrocytes can be demonstrated by reviewing oligodendrocyte-specific genes such as the *Myelin basic protein* gene (Mbp). Finally, microglia can be demonstrated by reviewing microglia-specific genes such as *Chemokine C-X3-C receptor 1* (Cx3cr1). An extensive list of glial subtype-specific genes was recently described in Lein et al., (2007). Review of the distribution of glial cells in the brain is extremely useful for the purposes of demonstrating grey and white matter. Specifically, review of oligodendrocyte distribution which is found in white matter areas such as the corpus callosum reveals the important role these cells play in axonal myelination. Figure 2 shows the spatial distribution of the oligodendrocyte-specific gene Mbp taken from the ABA. Higher magnification demonstrates the robust presence these cells in the corpus callosum (arrow).

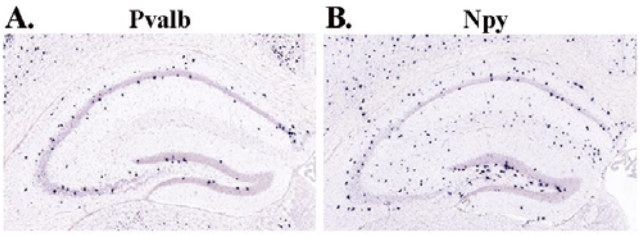

Figure 4. Revealing subtype-specific GABAergic neurons in the hippocampus by examination of expression of *Parvalbumin* (Pvalb; left) and *Neuropeptide Y* (Npy; right). Note Pvalb expression is limited to cells in the cell body layers, while Npy expression can be found throughout the hippocampus.

Using the ABA to demonstrate neuronal diversity in the brain

Neurons in the brain represent a heterogeneous cell population which can be subdivided according to several classification schemes. This feature of neuronal diversity can be difficult to demonstrate in laboratory-based classes without extensive tissue samples. The ABA can be exploited to reveal neurons that use different transmitter systems simply by searching for genes encoding specific molecules found in the synthesizing pathway for a given neurotransmitter. For example, examination of tissue hybridized against *Glutamic acid decarboxylase* genes (GAD1, GAD2) will reveal the distribution of GABAergic neurons in the brain. A similar approach can be used to demonstrate neurons containing acetylcholine, serotonin, and the biogenic amines by reviewing tissue hybridized for *Choline acetyl-transferase* (Chat), *Trytophan hydroxylase* genes (Tph1, Tph2), and *Tyrosine hydroxylase* (Th) and *Dopamine β-hydroxylase* (Dbh), respectively. Using this approach, students readily appreciate the distribution, for example, of cholinergic neurons in the brainstem and basal forebrain or of dopaminergic neurons in the ventral tegmentum and substantial nigra. Figure 3 shows the spatial distribution of the GABA neuron-specific gene Gad1 taken from the ABA. Note robust expression in the thalamic reticular nucleus (arrow in *A*). Higher magnification (*B*) demonstrates the presence of GABA neurons scattered throughout multiple strata of the hippocampus.

GABAergic neurons are among the most heterogeneous neuronal type (reviewed in Wonders & Anderson, 2006). This diversity can be demonstrated by reviewing tissue in the ABA hybridized against genes known to distinguish different GABAergic subpopulations. These genes include the calcium binding protein genes, *Parvalbumin* (Pvalb) and *Calretinin* (Calb2) as well as the neuropeptides genes, *Neuropeptide Y* (Npy), *Vasoactive intestinal polypeptide* (Vip), *Tachykinin* (Tac1), and *Somatostatin* (Sst). When reviewing material from the ABA for different GABAergic neuronal subtypes, students readily appreciate that the density of GABAergic neurons in different brain regions can vary widely. For example, in neocortex it can be easily seen that GABAergic neurons represent only a small population of cells (10-15%; White, 1989). In contrast, neurons in the thalamic reticular nucleus are exclusively GABAergic (Gad1+; see Figure 3) and also express Pvalb. The spatial distribution of different GABAergic subtypes can also be appreciated using the ABA. For example in the hippocampus, Pvalb and Sst-containing neurons are found in and close to the granule-cell and pyramidal-cell layers (stratum pyramidale and stratum oriens). In contrast, Npy-expressing cells are found in all strata including dendritic layers (stratum lacunosum-moleculare, stratum radiatum; Figure 4).

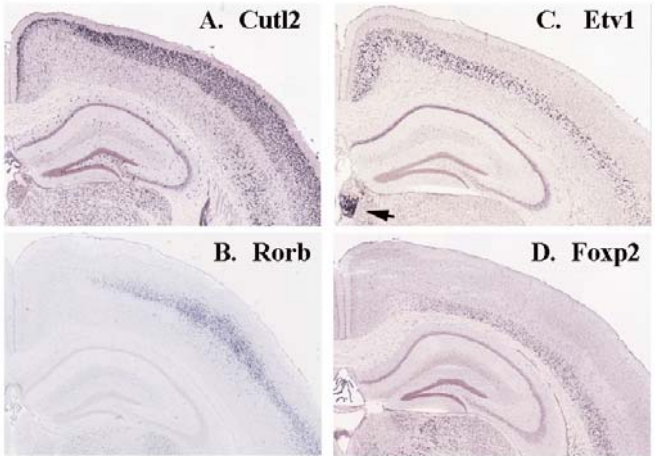

Figure 5. Demonstrating neocortical laminar networks with layer-specific gene expression. *A. Cut-like 2* (Cutl2) expression is concentrated in layers II/III while *RAR-related orphan receptor beta* (Rorb) expression (*B*) is found in layer IV. *Ets variant gene 1* (Etv1) expression (*C*) is found in layer V while *Forkhead box P2* (Foxp2) expression (*D*) is found in layer VI. Note strong expression of Etv1 in the medial habenula (arrow in *B*) but not Cutl2, Rorb, or Foxp2.

These demonstrations are intended to show how neuronal classification schemes can be multi-leveled. For example, neurons can be classified according to neurotransmitter used (e.g. GABA, glutamate). This criteria distinguishes neurons in general, all of which express Tubulin β3 (Figure 1), from those that use GABA, such as those cells that express Gad1 (Figure 3).

Therefore, when instructors and students review material found in the ABA for these two genes (Tubulin β3 and Gad1) this first-tier classification scheme is reinforced and students appreciate what a small percentage of GABAergic neurons are actually found in many areas such as in neocortex and hippocampus (compare Figure 1*B* with Figure 3*B*). Importantly, while only constituting a small percentage of cells in the brain, GABAergic neurons can be further subdivided according to a number of other genes. Therefore, when instructors and students review material found in the ABA for these GABAergic subtype-specific genes like Pvalb and Npy (Figure 4), this second-tier classification scheme is reinforced and students appreciate, for example, what a small percentage of GABAergic neurons also express Pvalb.

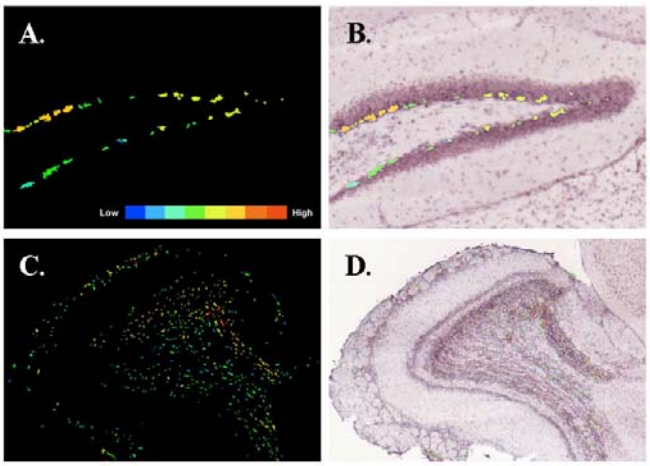

Figure 6. *Doublecortin* (Dcx) expression reveals the distribution of newly-generated neurons in the adult dentate gyrus (*A*, *B*) and olfactory bulb (*C, D*). Colorimetric analyses demonstrate Dcx expression levels (*A*). Newly generated neurons are found primarily in the subgranular layers of the dentate gyrus but widely distributed throughout the olfactory bulb. *B* and *D* are overlayed photomicrographs of colorimetric and hybridization data from ABA).

Demonstration of additional neuronal networks and subpopulations by cell-type specific gene expression

The ABA can be used to demonstrate virtually any cell-type or neuronal network that exhibits gene expression specificity. For example, neurons found in similar layers of neocortex share similar efferent projection patterns as well as gene expression profiles. The birthdates of neocortical neurons can also be revealed using layer-specific gene expression. This approach relies on the finding that individual neuronal lamina are made up of neurons with similar birthdates and that early-born neurons come to populate deep layers while subsequently generated neurons populate progressively more superficial layers (Angevine and Sidman, 1961; Rakic, 1974). Thus, examination of neocortical neurons containing the *Wolfram syndrome 1* gene (Wfs1; data not shown) reveals the exclusive expression of this gene in layer II neurons, which are among the latest cortical neurons to be born. Likewise, layer VI neurons, which are among the earliest neurons born during development, can be demonstrated by expression of *Forkhead box P2* (Foxp2). Using layer-specific gene expression, we have been able to effectively teach the principles of neocortical organization and development. Figure 5 contains representative photomicrographs taken from the ABA demonstrating layer-specific gene expression in neocortex.

Unlike neocortical neurons which are generated *in utero* (Rakic, 2002), granule cells in the dentate gyrus and periglomerular cells and granule cells in the olfactory bulb are continuously generated in the mammalian brain (Kempermann, 2005). The discovery of adult neurogenesis has led to much investigation of the potential of newly-generated neurons to treat neurodenerative disorders such Alzheimer's and Parkinson's diseases. The ABA can be used to demonstrate the presence of newly-generated neurons in the adult brain simply be examining tissue hybridized for the *Doublecortin* gene (Dcx; Rao and Shetty, 2004; Couillard-Despres et al., 2005). Figure 6 illustrates data from the ABA demonstrating Dcx expression in the dentate gyrus and olfactory bulb. Using this approach, students can come to appreciate the distribution of these important "new" neurons.

Regulation of the sleep/wake cycle is under powerful control of a small population of cells found in the hypothalamus which release hypocretin (reviewed in Sakurai, 2007). We used the ABA to demonstrate to students the spatial distribution of neurons containing Hcrt. Figure 7 illustrates Hcrt containing cells found in brains cut along sagittal (*A*) and coronal (*B*) planes. Magnification of photomicrographs using the zoom tool reveals individual Hcrt cells.

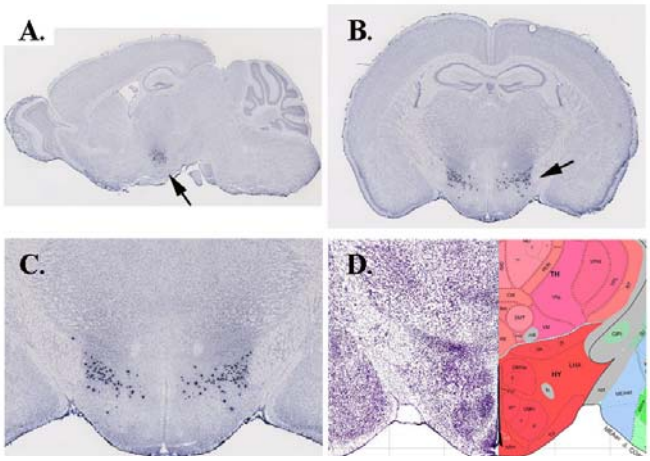

Figure 7. Revealing sleep/wake promoting neuronal networks in the hypothalamus by examination of expression of *Hypocretin* (Hcrt) containing cells (Arrows). Sagital *(A)* and coronal *(B-D)* photomicrographs taken from the ABA. *C*. Higher magnification of *B* reveals individual Hcrt-containing neurons. *D.* Associated image at the rosto-caudal position as in *C* taken from coronal Reference Atlas.

Using the Anatomic Search and Fine Structure Annotation tools to reveal cytoarchitectonic-specific gene expression

The Anatomic Search and Fine Structure Annotation tools found in the ABA provide a list of tissue hybridized for genes with cytoarchitectonic-specific expression. That is,

genes with identified expression in 36 different brain regions are listed including the thalamus, cerebellum, pons, etc. Therefore, it is not necessary to know genes found in these regions *a priori*. Using these search tools we have been able to demonstrate to students, for example, that the hippocampal subfields (dentate gyrus, CA3, CA1) can be divided according to unique gene expression patterns. Figure 8 illustrates region-specific gene expression in the lateral septum (*A*), locus ceruleus (arrow in *B*), facial motor nucleus (*C*, arrowhead in *D*) revealed by using the *Anatomic Search* and *Fine Structure Annotation* tools found in the ABA.

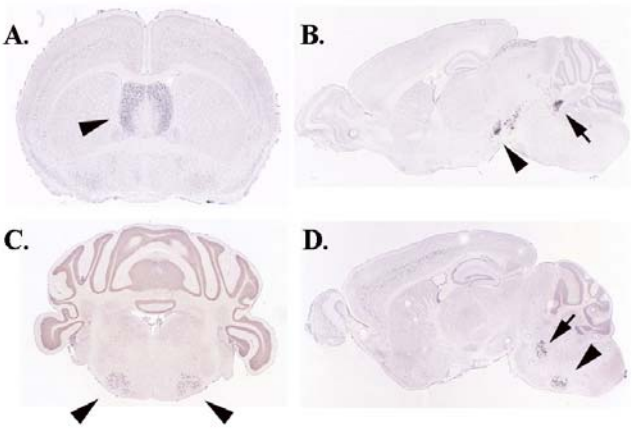

Figure 8. Region-specific gene expression using *Anatomic Search* and *Fine Structure Search* tools. *A*. Expression of *Transient receptor potential cation channel, subfamily C, member 4* (Trpc4) delineates the lateral septum. *B*. Expression of *Cholinergic receptor, nicotinic, alpha polypeptide 6* (Chrna6) delineates the locus ceruleus (arrow) and substantia nigra (arrowhead). *C*. Expression of *Heat shock protein 1* (Hspb1) delineates the facial motor nucleus. *D*. Expression of *Layilin* (Layn) delineates the facial motor nucleus (arrowhead) as well as the motor nucleus of the trigeminal ganglion.

Conclusions: The Allen Brain Atlas as a teaching tool in the teaching lab or lecture hall

In the present report we have described a strategy for using the ABA as a teaching tool in the lab in order to teach fundamental principles of neuroanatomy. As described above, instructors and students can navigate through the ABA and use gene expression patterns to reveal 1) cytoarchitectonic division in the brain, 2) cellular diversity (neurons vs. glia), 3) neuronal diversity (GABAergic, cholinergic, etc.), 4) glial cell diversity (astrocytes, oligodendrocytes, microglia), and 5) neuronal networks (individual layers of neocortex and subfields of hippocampus).

The ABA can be used in both lecture and laboratory-based classes. Instructors can load one or more images from the ABA for a given gene, instructing students how to use the ABA, and can review with students several points. First, any relevant information of the function of that particular gene should be discussed. Using some of the genes described above as examples, instructors can remind students that Mbp is the gene responsible for the synthesis of myelin-basic-protein which is made by oligodendrocytes and which serves as an insulator for axons. Likewise, instructors can remind students that Gad1 is the gene responsible for the synthesis of glutamic-acid-decarboxylase, an important component of the GABA synthesis pathway. Second, instructors should discuss the spatial expression profile of that gene with students, highlighting areas with high and low expression levels. For example, in the case of Mbp (Figure 2) and Gad1 (Figure 3), areas with high expression levels that might be discussed include the corpus callosum and the reticular nucleus of the thalamus, respectively. A detailed description of the spatial expression profile of a gene can be achieved by viewing micrographs from both coronal and sagittal sections as well as using the “zoom” tools. Finally, instructors should discuss the known function of those neural structures that demonstrate expression of that gene, highlighting any available data such as from reports of clinical populations following injury/insult or from lesion studies in animal models. In the case of Mbp and the high level of expression found in the corpus callosum, this would entail review of the important role of the callosum in interhemispheric transfer of neural information (reviewed in Bloom and Hynd, 2005). Discussion related to the callosum might also include studies done on split-brain patients (reviewed in Gazzaniga, 2005) or humans with callosal agenesis (reviewed in Kamnasaran, 2005).

These demonstrations can also be easily adapted into exercises that students can work on in groups or independently. Equipped with multiple computers in the teaching laboratory, we have previously given our students a list of genes which they use to search through the ABA. Students are asked to examine sagittal and/or coronal photomicrographs and document where they observe expression. This list included genes with similar cellular or regional expression patterns as well as genes with very different expression patterns. Because they are not told *a priori* where expression is located, students see these assignments as a challenge to find those areas with expression. After completing the database search, students can present and discuss their observations formally with a presentation in front of the class using representative images from the ABA (using the projector).

Because the ABA is found on the internet and access to this database is available 24 hours a day, instructors can also create take-home assignments for students, requiring that they perform gene or anatomical region searches. Our experience is that students enjoy the interactive nature of the ABA and learn a lot from these exercises. As the number of genes that are functionally characterized increases, so will the utility of the ABA to serve as an important teaching tool.

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