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DOPAMINE AND SEROTININ SUBRECEPTOR DISTRIBUTION IN THE MURINE HIPPOCAMPUS

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1.0 Introduction

Neurogenesis is the generation of new neurons from stem cells, and is ubiquitous and prominent during prenatal and early postnatal development. In the adult the rate and location of ongoing neurogenesis is far more restricted. Through ligand and lesion studies of the dopaminergic and serotonergic systems, it has been established a clear connection between dopaminergic and serotonergic regulation and adult hippocampal neurogenesis (Banasr, Hery, Printemps, & Daszuta, 2004; Borta & Hoglinger, 2007; Brezun & Daszuta, 1999; Djavadian, 2004). Several studies have found a relationship between adult neurogenesis and treatment of mood related illnesses, such as psychosis, depression and schizophrenia or recreational drug use such as cocaine. Common for all of the above is their influence on neuromodulatory systems in the brain, including the dopaminergic and serotonergic systems. In addition to an effect on neurogenesis, both dopamine and serotonin have clear effects on hippocampus dependant memory formation (Bethus, Tse, & Morris, 2010; Jay, 2003; King, Marsden, & Fone, 2008; Ogren et al., 2008). This is not surprising, as they are centrally involved in reward circuitry and value judgments, both of which are central aspects of learning. Both adult neurogenesis and memory formation are two specialities of the Hippocampus.

1.1 Hippocampus

The hippocampus has various functions. It is involved in memory formation and transfer to the cerebral cortex (Deng, Saxe, Gallina, & Gage, 2009). Through special place-sensitive neurons it is central for spatial navigation, and it is involved in emotional processing with its caudal connections to the amygdala, and its regulation of the HPA-axis.

1.1.1 Anatomy

The hippocampal system is divided into hippocampal (hippocampal formation) and parahippocampal (medial entorhinal cortex) subsections. The hippocampal formation is a c shaped structure stretching in a rostrodorsal to caudal-ventral orientation in the lateral temporal lobe (van Strien, Cappaert, & Witter, 2009). It has three main subregions, the hippocampus proper consisting of the cornu ammonis (CA1, CA2 and CA3), the dentate gyrus and the subiculum (van Strien et al., 2009). The parahippocampal region consists of the entorhinal cortex, perirhinal areas 35 and 36, postrhinal cortex, the presubiculum and the parasubiculum (Kerr, Agster, Furtak, & Burwell, 2007).

The hippocampal formation has three layers with a central principal neuron layer surrounded by polymorphic layers (van Strien et al., 2009). The principal cell layer contains the cell bodies of the granular cells of the dentate gyrus and the pyramidal cells of the cornu ammonis (van Strien et al., 2009).

The polymorphic layers surrounding the granular cell layer is the molecular layer and the hilus, or CA4 (van Strien et al., 2009). The polymorphic layers of the cornu ammonis regions is on the outside of the pyramidal layer divided into the stratum oriens and the alveus. On the inside of the pyramidal layer the CA3 has a unique area called the stratum lucidum that contain the mossy fiber input from the dentate gyrus. Further out is the stratum radiatum and the stratum lacunosum moleculare (van Strien et al., 2009). The polymorphic layers contain interneuron cell bodies and neurites of principal cells and interneurons.

1.1.2 Connectivity

The entorhinal cortex functions as a bidirectional gateway between the neocortex and the hippocampus (Kerr et al., 2007). The connectivity between regions is primarily feed forward and constitutes a polysynaptic circuit commonly called the trisynaptic loop. The primary excitatory input to the hippocampus stems from entorhinal cortex innervations through the perforant path which projects to the dentate gyrus and CA3 (Kerr et al., 2007).

The mossy fibers are the major efferent path from DG and project to dentate gyrus interneurons and CA3 inter- and pyramidal neurons (Mongiat, Esposito, Lombardi, & Schinder, 2009; van Strien et al., 2009). CA3 pyramidal cells project to CA1 pyra-midal cells through the Schaffer collateral. CA1 and the subiculum project to the deep layers of entorhonal cortex, closing the trisynaptc loop (Kerr et al., 2007). The deep layers project out to the cortex including the superficial entorhinal cortex. Various parts of the EC project to the

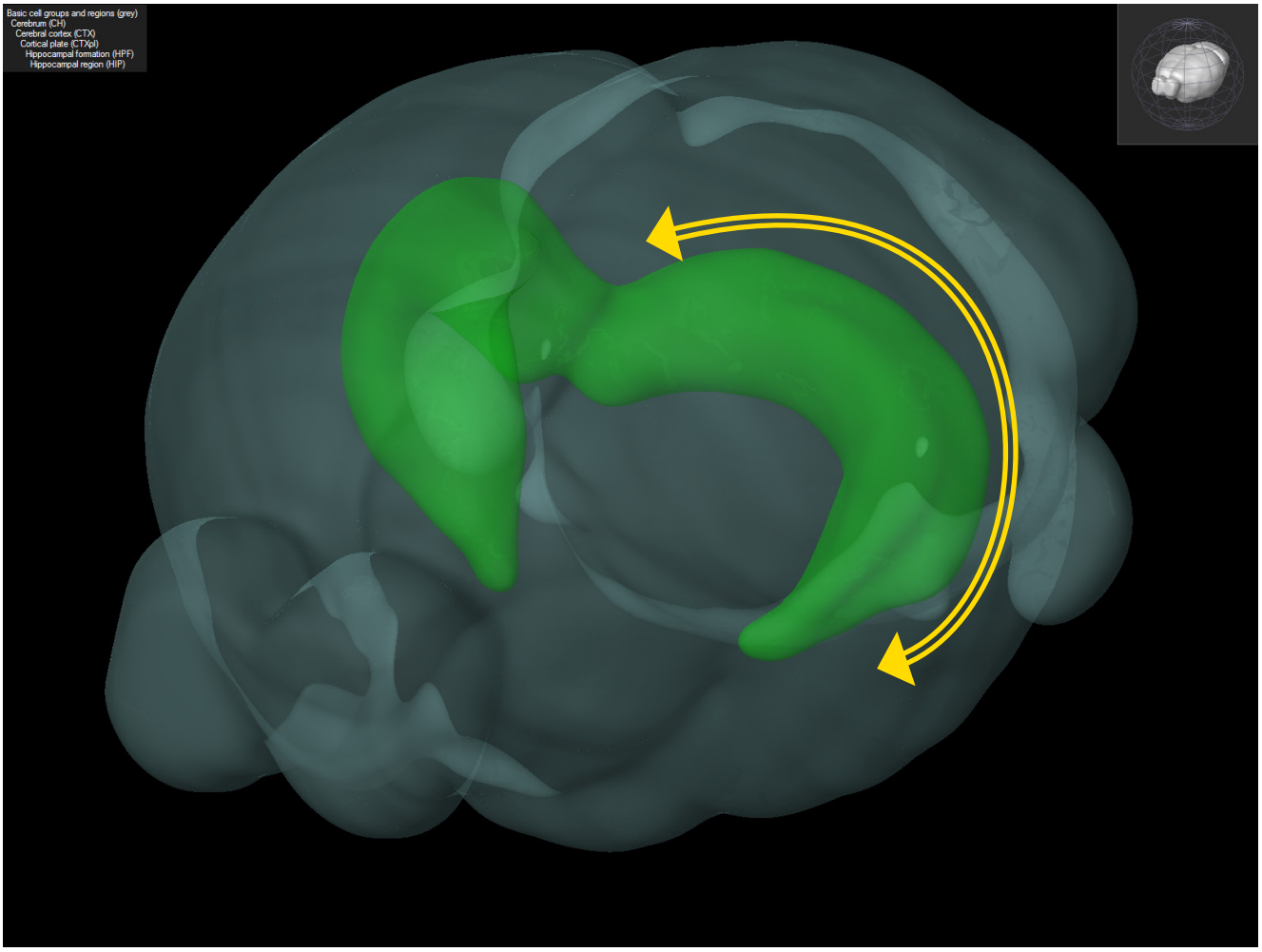


Fig 1. Created in Brain Explorer 2 ©2012 Allen Institute for Brain Science. 3D model of the Hippocampal system in the mouse brain. Arrow shows the orientation in the brain.

DG, CA3, CA1 and subiculum directly (Kerr et al., 2007).

1.1.3 Neurogenesis

Before the discovery of adult neurogenesis it was assumed that all neurons generated in embryogenesis, and that the population steadily declined with age. Adult neurogenesis, upon its discovery in the avian songbird homologue of the hippocampus, soon became a popular field of study. This was both due to the revisal of our understanding of the brain as a more plastic organ than previously thought, as well as the potential for clinical application in the regeneration and restoration of damaged and aged brains. Neurogenesis in mammals is found in two brain areas, the olfactory bulb and the dentate gyrus of the hippocampus (Aimone, Deng, & Gage, 2010). The stem cells of the subgranular zone (SGZ) develop into granule cells in the granule cell layer of the dentate gyrus (Treves, Tashiro, Witter, & Moser, 2008). In the SGZ stem cells differentiate into progenitor cells which mature into granule cells and migrate a short distance into the granular layer of the DG (Deng et al., 2009). The rate of neurogenesis varies between different species. In mice and rats the rate of survival of newborn granule cells in the dentate gyrus is 1000-3000 per day, i.e. approximately one thousandth of the total population of DG granule cells being re-

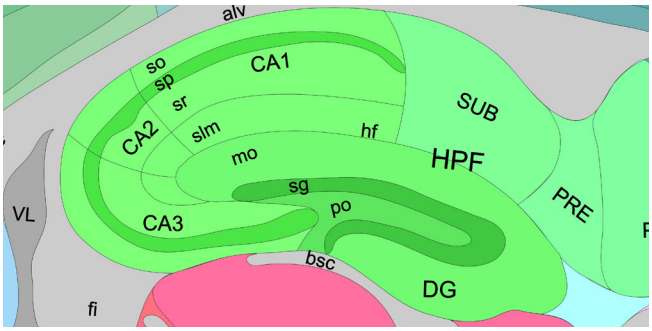


Fig 2. Anatomical reference atlas of the saggital section of the dorsal mouse Hippocampus. ©2012 Allen Institute for Brain Science.

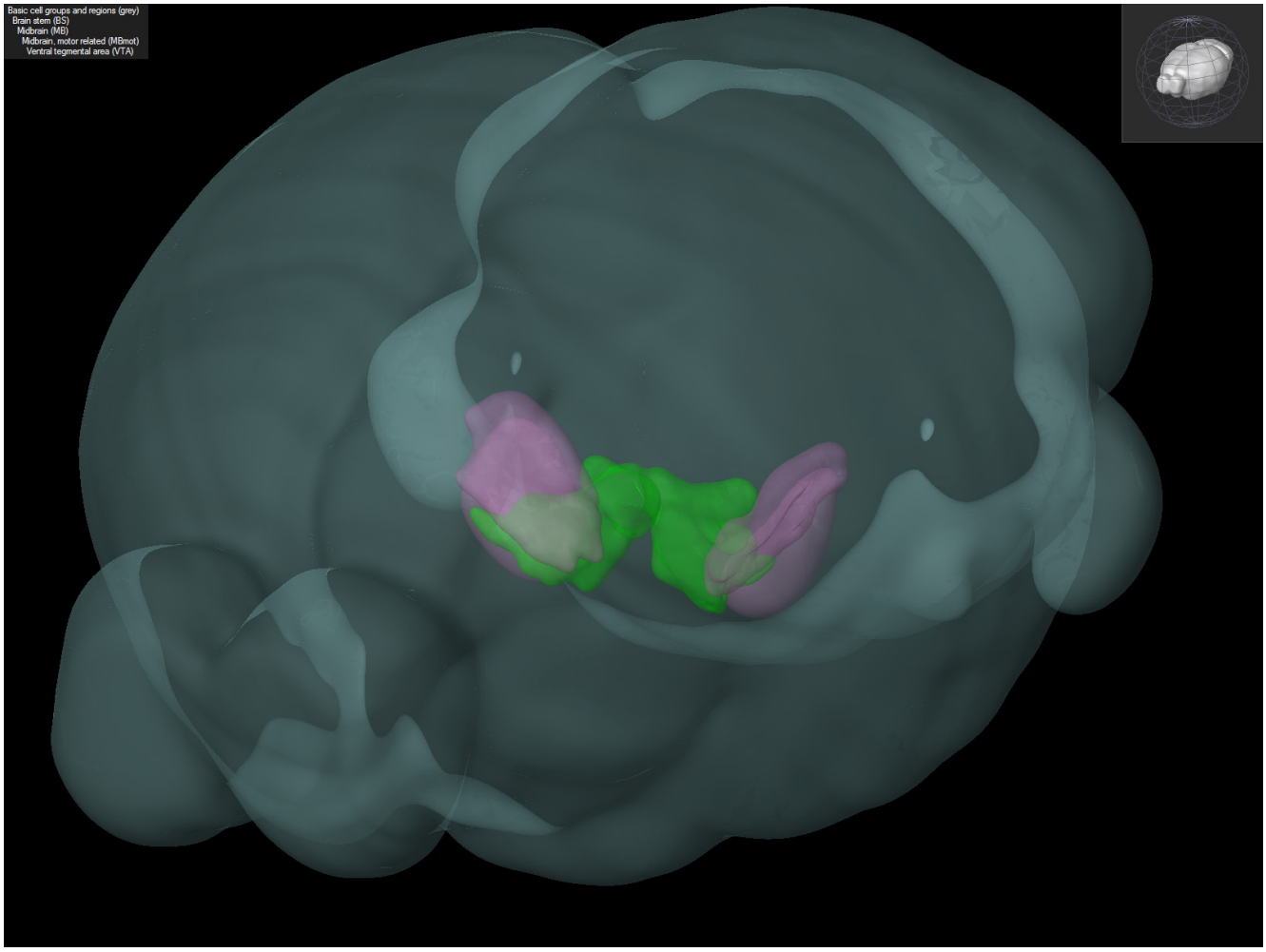


Fig 3. Created in Brain Explorer 2 ©2012 Allen Institute for Brain Science. 3D model of the dopamine system in the mouse brain. VTA: Ventral tegmental area (bright green),SNc: substantia nigra pars compacta, SNr: substantia nigra pars reticulata (both in purple).

placed each day.(Djavadian, 2004). As the granule cell layer remains fairly constant throughout the individuals' age, the cell population is fairly stable and the rate of cell death corresponds to the rate of production of granule cells with adult neuronal properties. In the dentate gyrus the adult born newborn neurons are believed to play a role in memory formation (Aimone et al., 2010). Whether it is involved in encoding, consolidation, retrieval or the transference of the memory from the hippocampus to the neocortex is not yet clear.

1.2 Dopamine

Dopamine is a monoamine neurotransmitter of the cathecholamine family. It is functionally involved in mood regulation, reward circuitry, voluntary muscle control, punishment, sleep, attention, working memory and learning (Jay, 2003; Missale, Nash, Robinson, Jaber, & Caron, 1998) Dopamine is also involved in reward prediction and surprise detection and involved in effort invested in obtaining rewards (Shohamy & Adcock, 2010). The dopamine system responds to novelty, excreting DA quickly and precisely. DA receptor activation influence plasticity, and may create or fixate the plasticity of glutaminergic activity (Jay, 2003).

1.2.1 Anatomy

The primary dopaminergic nuclei are the susbtantia nigra pars compacta, pars reticulata (fig. pink areas) and the ventral tegmental area (fig. bright green) of the midbrain, from where they project to large parts of the mammalian brain. In addition, there exists a small cluster of dopaminergic neurons in the arcuate nucleus of the hypothalamus. The dopamine system can be divided by the main pathways that innervate and regulate functionally similar areas. The mesolimbic pathway projects from the VTA to the hippocampus and amy-

gdala (Yang, Arnold, Habas, Hetman, & Hagg, 2008). The Ventral Tegmental area projects to the hippocampus as well as the substantia nigra, from the A10, A9 and A8 neuron groups (A10 is the VTA, A9 is the SN) (Jay, 2003). The A10, A9 and A8 neuron groups form symmetric synapses on dentate granule cells, CA3 pyramidal cells and CA1 pyramidal cells (Romo-Parra, Aceves, & Gutierrez, 2005). The most major innervation is of the subiculum and the CA1. There is a more prominent dopaminergic innervation of the ventral part of the hippocampus compared to the dorsal. The dorsal hippocampus receives innervations from the ventral tegmental area (Romo-Parra et al., 2005).

1.2.2 Dopamine receptor families

The dopamine sensitive receptors are divided into two families, based on structural homology and effects on intracellular signaling cascades (Borta & Hoglinger, 2007). So far five dopamine receptor subtypes have been found in mammals; in addition there exist cross-species sub-receptor isoforms and alternate splicing of some of the members of the D2-family. All of the receptor subtypes are guanine nucleotide-binding protein (G-protein) coupled receptors, with a seven transmembrane alfa-helical structure (Missale et al., 1998). The transmembrane areas show little variation between receptor types. The area that shows the least structural homology is the third intracellular loop, which varies in length dependent upon the number of repeat sequences (Missale et al., 1998). Most available probes and antibodies target this part of the receptors (Missale et al., 1998)

1.2.3 D1-like family

The D1-like receptor family consists of the Dopamine D1-receptor and the D5-receptor. The receptors are Gs-coupled (Sunahara et al., 1991; Zhou et al., 1990). Activation of this receptor family by dopamine lead to increased intracellular cAMP through adenylate cyclase activation. Receptor activation usually has an excitatory effect. The D1 sub-receptor is the most widespread in the brain (Fremeau et al., 1991). The D1-like family receptors contain no introns in their coding region (Missale et al., 1998). The D1 receptor has no introns in its coding region, and as such does not have functional isoforms (Mansour et al., 1992; Missale et al., 1998). The distribution of the D1 receptor subtype is far greater than the D5 receptor subtype, however, the affinity of the target ligand dopamine is ten times greater in D5 than in D1(Sunahara et al., 1991).

1.2.4 D2-like family

The D2-like receptor subfamily consists of the D2, D3 and D4 receptor subtypes. The D2-like receptor family is Gi/Go-coupled and decrease intracellular levels of cAMP through the inhibition of adenylate cyclase activity (Missale et al., 1998). Of the D2-like sub-receptor family, the most abundant is the D2 sub-receptor (Brouwer, Van Dijken, Ruiters, Van Willigen, & Ter Horst, 1992). As is the case with the D1-like sub-receptor family, the lacking abundance of the D3 and D4 receptors compared to the D2 receptor is compensated by the higher affinity for Dopamine (Sokoloff, Giros, Martres, Bouthenet, & Schwartz, 1990; Van Tol et al., 1991).

In contrast to the D1-like family there are introns in the coding region of the D2-like receptors (Brouwer et al., 1992). This gives functional ligand binding long and short isoforms of D2 in many mammals, and of D3 in mice (Missale et al., 1998). In mice a functional non-binding receptor also exists (Richtand et al., 2010).

1.3 Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter synthesized from the amino acid L-tryptophan. The name serotonin is derived from serum in which it was first isolated, where it serves as a vasoconstrictor. Serotonin is an old and ubiquitous signaling molecule that has varied roles in different species and different tissues. In the central nervous system serotonin is known to be involved in the regulation of mood, sleep, learning, memory and appetite (Barnes & Sharp, 1999).

While the serotonergic system innervates nearly every brain system, there is evidence that the different nuclei and subregions innervate functionally similar regions and are capable of specific transmitter release to functionally relevant areas(Barnes & Sharp, 1999). The dorsal raphe nuclei project to the brain via the medial forebrain bundle, and innervate nearly every brain structure (Djavadian, 2004). One such area is in-

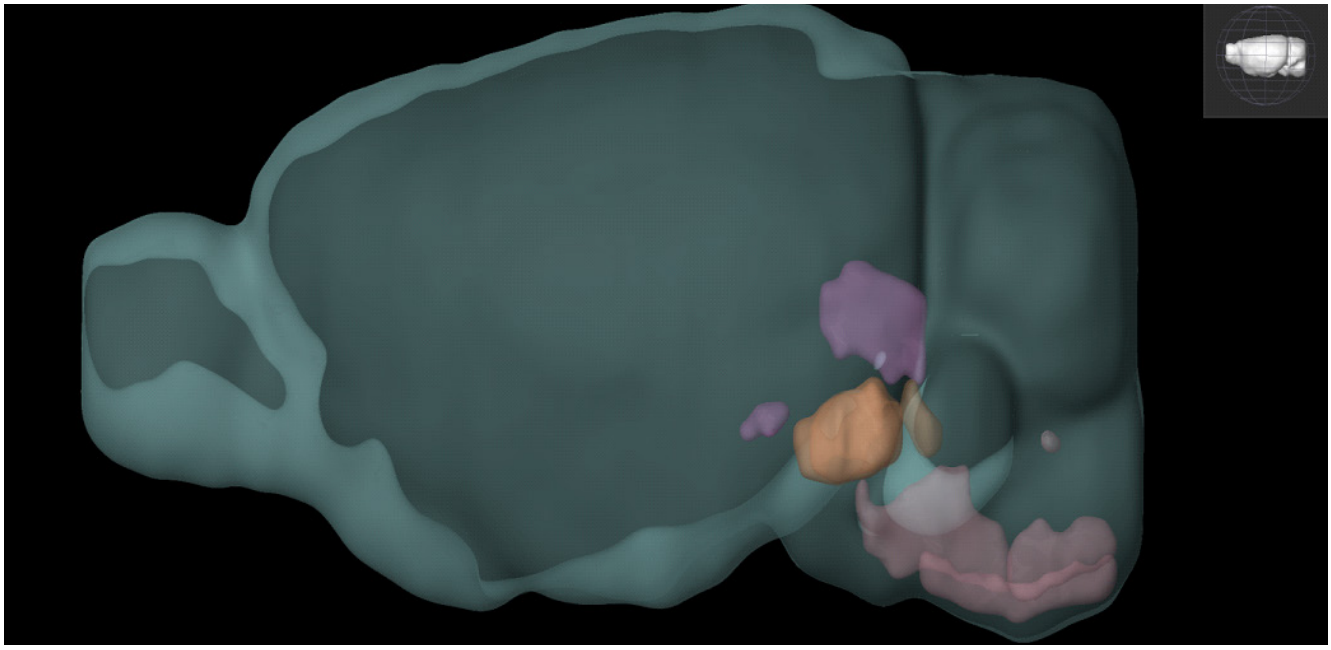


Fig 4. Created in Brain Explorer 2 ©2012 Allen Institute for Brain Science. 3D model of the serotonergic raphe nuclei in the mouse brain.

volved in memory modulation through the limbic system where the hippocampus receive dense innervation (Djavadian, 2004).

1.3.1 Anatomy of the serotonergic system

The primary nuclei involved in Serotonin synthesis and secretion in the brain are the nine raphe nuclei, which lie along the brain stem. Each nucleus has functionally distinct innervations, and they can be grouped by projection pattern and function. The nuclei are denoted B1-B9. The caudal nuclei project mainly to the cerebellum and the brain-stem (B1-B3). The median raphe nuclei (B5 and B8) have both brain and brain-stem projections. The dorsal raphe nuclei (B6 and B7) project to various cortical and sub-cortical regions, including the hippocampus. The dorsal raphe projects to the hippocampus, the DG and the CA3 (Jay, 2003).

In the hippocampus 80-90% of the serotonergic projections do not have synaptic contact with target cells, but rely on extrasynaptic receptor activation (Vizi & Kiss, 1998). Projections from the dorsal raphe to the hippocampus do not make synaptic contacts (Vizi & Kiss, 1998). Approximately 21% of the projections from the median raphe make synaptic contact onto hippocampal GABAergic interneurons (Vizi & Kiss, 1998).

1.3.2 Receptors

There are seventeen registered receptor subtypes sensitive to serotonin. These are spread across seven different receptor families dictated by molecular and genetic similarities and intracellular effects of ligand binding. All families are metabotropic except for the ionotropic HTR3 family. The metabotropic serotonin receptors are seven transmembrane alfa-helical G protein-coupled receptors (Barnes & Sharp, 1999).

1.4 Serotonergic and dopaminergic effects on hippocampus-dependant processes

There are several findings that indicate a connection between dopamine and serotonin mediated processes and neurogenesis. Serotonin-regulated emotional states influence the survival of newborn neurons (Aimone et al., 2010). Administration of dopamine and serotonin receptor selective ligands to treat anxiety leads to an increased amount of cells expressing BrdU, thymidine analogue used to detect replicating cells (Djavadian, 2004). In turn, an intact neurogenesis is required to rescue an animal from anxious or depressive phenotype after psychosocial stress exposure (Schloesser, Lehmann, Martinowich, Manji, & Herkenham, 2010). Ablation of neurogenesis in rats leads to increased dependence behavior when the rats self administer

cocaine, as well as a large impairment of the extinction learning of the cocaine addiction (Noonan, Bulin, Fuller, & Eisch, 2010). Depletion of dopamine in the hippocampus leads to a transient increase in neurogenesis (Park & Enikolopov, 2010), while depletion of serotonin leads to a decrease in neurogenesis (Brezun & Daszuta, 1999). In Parkinsons patients proliferation of the subventricular zone and the dentate gyrus is decreased (Winner et al., 2009). D2 or D2-like receptors have been linked to the pathophysiology of Parkinsons disease (Chen, Qin, Szele, Bai, & Weiss, 1991). With administration of a serotonergic antidepressant, previously mature dentate gyrus granule cells seem to regain immature properties (Kobayashi et al., 2010). Whether the regulation of hippocampal processes by the neuromodulatory systems is direct or indirect is not yet know. Most probably there is a combination of both, or it is dependent upon the process under study. Dopamine D1.like control of neurogenesis has been found to work indirectly through regulation of the GABAa receptor (Goffin, Aarum, Schroeder, Jovanovic, & Chuang, 2008). D1 has also been found to be involved in LTP and spatial learning in the hippocampus, while the D5 receptor has not (Granado et al., 2008).

Increased motor activity stimulates neurogenesis in the dentate gyrus, but not in the subventricular zone (Winner et al., 2009). Dopamine depletion inhibits progenitor cell proliferation (Ming & Song, 2005). Another study found that D3 may be involved in neurogenesis of rats, but not in mice (Baker, Baker, & Hagg, 2005). In a mouse model for Parkinsons, the depletion of dopamine lead to a transient increase in neurogenesis in the dentate gyrus subventricular zone (Park & Enikolopov, 2010). The D2-like family receptor activation lead to decreased proliferation (Borta & Hoglinger, 2007). D1 seems to have the exact opposite effect where its stimulation inhibits progression through the cell cycle (Baker et al., 2005), and activate proliferation of precursor cells (Borta & Hoglinger, 2007). Stimulation of D2 in rats has an inhibitory effect on granule cell excitability, an important factor for the activity dependant survival of newborn neurons (Romo-Parra et al., 2005).

The full effect of SSRIs on depression are only realized after approximately four weeks. This has led to speculation as to whether the full antidepressant effects of SSRIs are dependent upon hippocampal neurogenesis, as these cells are functionally integrated after an approximately four week period (Morales & Backman, 2002). The effect on neurogenesis is subtype specific, where activation of the 5-htr1a leads to an increase of progenitor cell proliferation in the subventricular zone of mice, while activation of the 5-htr2a decreases proliferation (Platel, Stamboulian, Nguyen, & Bordey, 2010).

1.5 The effect of Serotonin and Dopamine on memory

Stimulation of the D1 receptor has clear effects of various forms of memory, and D1 is the main regulator of memory in monkeys (Jay, 2003). Activation of the D1 receptor has been shown to affect both LTD and LTP in the hippocampus and the prefrontal cortex (Granado et al., 2008). In D1 receptor knock-out mice, the formation of Long LTP is impaired and LTP-induced arc and zif268 expression in the CA1 can be blocked by using a D1 antagonist (Granado et al., 2008; Jay, 2003). D1 may facilitate both LTP and LTD (Granado et al., 2008). Serotonin depletion in rats modulates LTP in the dentate gyrus (Kulla & Manahan-Vaughan, 2000). In addition to an effect on LTP, an excessive or insufficient stimulation of the D1 receptor in the prefrontal cortex leads to an impairment that affects working memory (Granado et al., 2008). Activation of all D2-like receptors in the hippocampus in rats improves both encoding and retention in certain working memory tasks (Basile et al., 2006).

The neuromodulatory systems have wide projections to large parts of the brain, and their activation has population-wide effects. The neuromodulatory nuclei contain neuronal subgroups that modulate functionally similar areas, giving a functionally, if not cellular, precise modulation. This means that the neuromodulatory systems have the specificity required to induce a given effect in a given neuronal cell population. A central question in the relationship between the neuromodulatory systems and the hippocampus is whether their effect is a direct regulation of stem cells and progenitor cells, or whether the effect is a network regulation of activity. With precise knowledge of sub-receptor

expression in the hippocampus, one may target both direct and indirect regulation and uncover their role in the hippocampal formation.

1.6 Inconsistencies in the subtype expression data

As seen above, there is ample evidence of a dopaminergic and serotonergic effect on all of the known functions in the hippocampus. As sub-receptor cloning became available, the strong link between serotonin, dopamine and the hippocampus became more apparent and more behavioral studies to determine their relationship was performed. What also became apparent was the great variety that existed in the sub-receptor expression itself, and in their posttranslational processing. Many of the receptors display a high degree of structural homology, making the generation of precise antibodies and mRNA probes more difficult. The Dopamine family has two receptor families, the D1-like family has no introns and thus no isoforms. In the D2-like family there are several introns and several functional isoforms within sub-receptor families, not all of them compound-binding. The serotonin receptors are divided in seven families, the third of these is ionotropic. Additionally the families have receptor isoforms, leading to a great variance in sub-receptor expression between tissues and animals. The receptor expression appear to have a certain degree of redundancy.

In addition some available ligands are not sufficiently specific to only activate the desired sub-receptor. These are not subtype specific, binding both to different receptor families within the same neurotransmitter, and to receptors of related neurotransmitters. This led to conflicting expression patterns in several sub-receptor, leading to some uncertainty as to which sub-receptors are functionally expressed in a given brain area.

1.6.1 Interspecies comparison

While most of the dopamine and serotonin sub-receptors are well-characterized in the rat brain, it is less so in the mouse brain. This has turned out to be functionally relevant, as both the dopamine and serotonin receptors show a great expression variety between species. Humans, guinea pigs, rats and mice show different expression patterns in several sub-receptors (Bruinvels et al., 1994; Ciliax et al., 2000; Khan et al., 2000).The administration of the D3 preferring agonist 7-OH DPAT stimulates proliferation in the subventricular zone of lesioned and unlesioned rats (Winner et al., 2009). In mice the increase in proliferation after D3 stimulation is not present (Kim et al., 2010). The variance between animals may also to a lesser extent be present between animals of the same species with different genotypes. The effect of dopamine stimulation on neurogenesis in the subventricular zone has been found to be dependent upon the strain of mouse used (Platel et al., 2010).

The lack of specificity of the methods previously used, and the high interspecies and brain region variance in receptor distribution and function make correct distribution classification difficult (Kim et al., 2010; Noain et al., 2006). The lack of specific antibodies made it necessary to rely on mRNA expression levels that does not reliably reflect the amount of functional receptors present at the cellular membrane, as seen in the spinal cord of D3 knockout mice where D3 mRNA is still expressed, but not translated to functional proteins (Zhu, Clemens, Sawchuk, & Hochman, 2008).

2.0 Aim of thesis

In studying the literature I found that there is a great discrepancy between the reported expression sites in different studies. Some of the discrepancies could be attributed to different methodology, and some could be attributed to interspecies differences. However, it became clear that the subtype specificity of the methodology employed was unreliable, leading to much discussion regarding which sub-receptors were expressed where. The varying results of commercial and self-cloned antibodies indicated a less than precise subtype specific binding, leading to a general halt in its use since the middle of the 2000s. Radioligand binding became more popular as compounds with a greater subtype specific affinity was developed, and are still used, often in combination with non-marker ligands that bind competing receptors. Transgenic bacterial artificial chromosome mouse lines coexpressing the enhanced green fluorescent protein (EGFP) with the target gene have been developed. The expression distribution in mice has been less studied in mice than in rats. Even fewer have studied the developmental expression of serotonin and

dopamine in mice, expression patterns that may indicate the regulatory role of neurotransmitters on adult neurogenesis. Both neurotransmitters are implicated in both neurogenesis, memory formation and emotional regulation. Since the distribution expression may be important for the regulation of hippocampal functionality, I will in this study use two publicly available mouse brain databases to determine the expression of serotonergic and dopaminergic sub-receptors in the adult and developing murine hippocampus.

The purpose of this thesis is:

1. To determine which dopamine and serotonin sub-receptors is expressed in the dentate gyrus, CA3, CA2 and CA1 during postnatal developmental stages, and in adult mouse brains.

2. To use the anatomical distribution pattern of stained cells in the Dentate Gyrus to determine which sub-receptors may be expressed on adult-born newborn neurons generated in the subgranular zone of the Dentate Gyrus.

3.0 Method

The method employed in this thesis is a study of the GENSAT database and the Allen Brain map project. In addition I have compared the expression levels of the neuromodulatory distributions in the HC with earlier ligand and staining studies of the different receptor subtypes. Due to the lack of specificity of earlier methods, the exact expression levels of the different subtypes of receptors have been difficult to map. As such the evidence presented here is an argument for a likely distribution of the various receptor subtypes.

3.1 Allen brain map

The Allen Brain Atlas (The Allen Institute for Brain Science, 2010) is an online database containing a wide selection of gene expression data in the adult and developing C57BL/6J mouse brain as well as in the adult human brain. The database is published by the Allen Institute for Brain Science. The database publishes digital pictures of brain slices treated with a non-radioactive digoxigenin based in situ hybridization (ISH) technique that label cells expressing target genes. The digoxigenin is in turn labeled by a horse radish peroxidase bound antibody. After an amplification process, the HRP react with a biotin coupled binding agent that is later stained with nitroblue tetrazolium. More details on probe development and tissue staining can be found here: http://help.brain-map.org/download/attachments/2818169/ABADataProductionProcesses.pdf?version=1&modificationDate=1319477154403

In addition to the above method, the developing mouse brain protocol uses a Feulgen-HP yellow DNA counterstain to increase the definition of the tissue, as young slices are more transparent than adult slices (Allen Institute for Brain Science, 2012). Further information on the generation of the developing mouse brain atlas can be found in the technical white paper: http://developingmouse.brain-map.org/docs/Overview.pdf

3.1.1 Animals

The mice used in the database are male C57BL/J6 from the Jackson Institute. I have searched the database for expression levels at ages adult (P56) and postnatal day 14 (P14). The day of birth is termed P0.

3.1.2 Brain Explorer v.2.

The Brain Explorer is an application that allows placing expression data of target genes in a 3D model of the adult and developing mouse brain. The resolution of the expression data is 200µm³, termed a "voxel". Each voxel has an averaged valued calculated from the expression intensity of all contained tissue. The voxels are colour coded according to expression intensity in a heat map, where red is strongest, and blue is weakest. The expression data is derived from the Allen Reference Atlas (ARA). I have used the Brain explorer as a tool to compare the staining intensity seen in slice images and the stated expression level to better get comparative results. The Brain Explorer was also used to generate three dimensional anatomical images of the serotonergic, dopaminergic and hippocampal region.

I have only used the database to support slice evaluation, and not as the primary dataset as there were some flaws with the database. The size of the voxel would sometimes lead to a subregion misnormer in small structures, making it less useful for the mouse hip-

pocampus. Furthermore the database does not exclude signals from tissue that is damaged or shrunk, sometimes showing strong staining intensity in unexpected/erronous regions. This can be obvious in staining distributions of coronal sections where there is great asymmetry in expression patterns between hemispheres. In monohemispheric sagittal sections, this can be more challenging. As such I used it to compare what was determined as different expression levels in nitroblue tetrazolium stained slices of the murine hippocampus.

3.2 GENSAT project

The Gene Expression Nervous System Atlas (GENSAT) is a publicly available project at Rockefeller University funded by the National Institute of Health (The Rockefeller University). It uses bacterial artificial chromosome (BAC) transgenic mouse lines co-expressing EGFP, tdTomato or Cre recombinase with the target genes. The reporter gene is inserted upstream from the start codon of the target gene. During transcription, both genes are controlled by the promoter and regulatory elements of the target gene (The Rockefeller University). The BAC-EGFP slides are treated with an antibody for EGFP, according to the protocol found here: http://www.GENSAT.org/HistologyProtocol.pdf. The use of an antibody instead of EGFP fluorescence increases the signal strength of the expression (The Rockefeller University).

3.2.1 Animals

The animals used in this study are aged mature (P42) and postnatal 7 days (P7). Day of birth is P0. The transgenic animals are produced according to this protocol: http://www.GENSAT.org/TransgenicProtocol.pdf.

3.3 Slice selection

I searched the GENSAT, Allen mouse brain atlas and Allen developing mouse brain atlas databases with the gene names as key words, and in case of unsuccessful results, dopamine or serotonin. When possible, I chose saggital sections of the dorsal/septal hippocampus. When saggital sections were not available, I used coronal sections. I used three sections from each image series, preferably in series. When large artifacts or damage to the hippocampus was present, I used other sections, but always of the dorsal/septal HC. I only used series whose distribution had been verified by GENSAT or had passed ABA quality control. At GENSAT the series are verified by agreement with literature, multiple lines matching, a matching of ISH data or any combination of these. At ABA the slides are examined by a quality control team, and released to the public if the quality is satisfactory. Each series is a collection of slices from the same specimen, treated with the same method.

3.3 Calculation of expression density

I have employed a binary system of expression where I have evaluated whether tissue shows expression or not. I have used both the staining intensity and the shape of the staining to determine whether a slice shows staining. I opted for a mostly conservative interpretation, excluding weak expressions or staining with a non-cellular shape. However, in some cases a weak staining would display subregion specificity in the molecular layers. In slices where there is no discernible expression, there is no such subregion specific coloration. I have in these cases been more liberal with determining expression.

To aid in determining expression I compared staining of colored pictures and grayscale pictures of the GENSAT database, to minimize the effect of color change between tissues. In the Allen database pictures, I used the original pictures and the available heatmap mask to compare expression levels. To ensure a concervative evalution, I excluded all stained cells that appeared blue/green in the heatmap.

The expression pattern was then determined in a six point scale, from single cells at one to ubiquitous at six. After coding for each hippocampal subregion, Excel was used to generate diagrams of the expression.

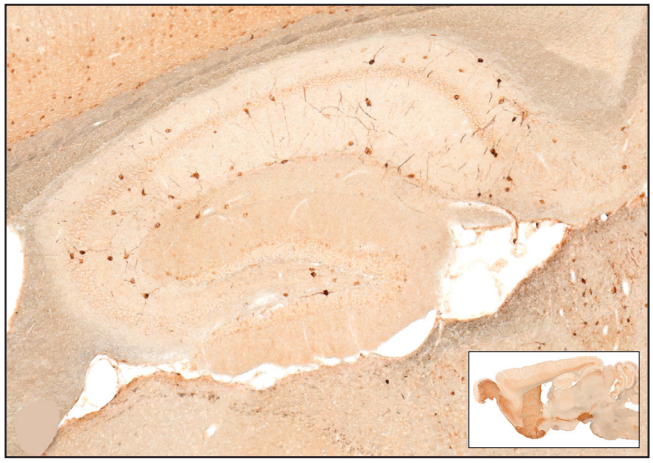
4.0 Results

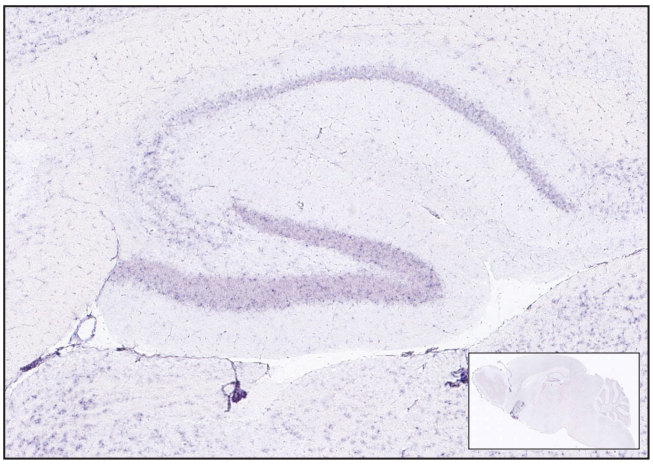
4.1 Dopamine

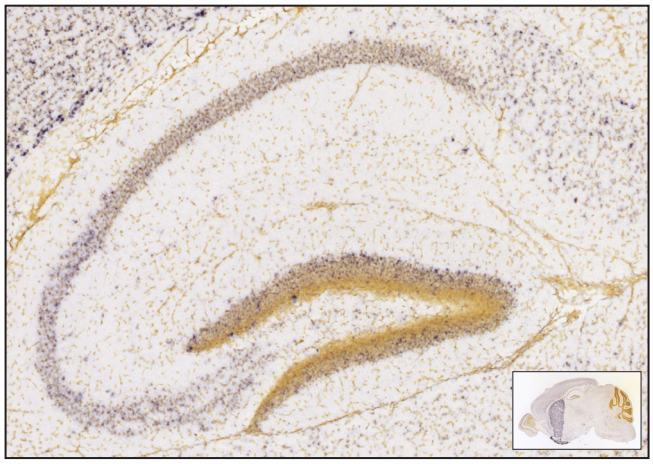
The results are illustrated and explained on the following pages, denoted by D.

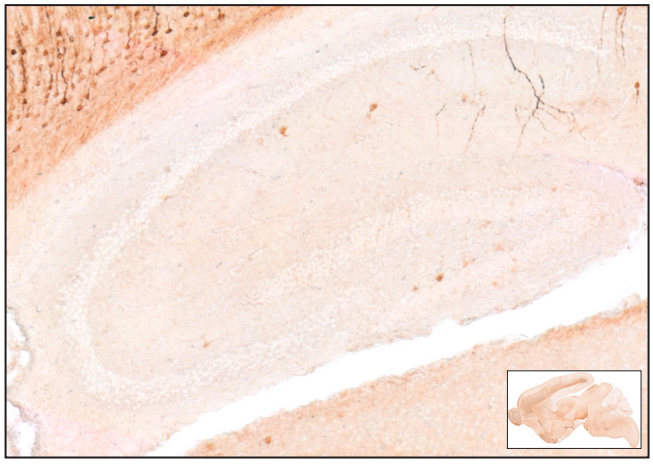
4.2 Serotinin

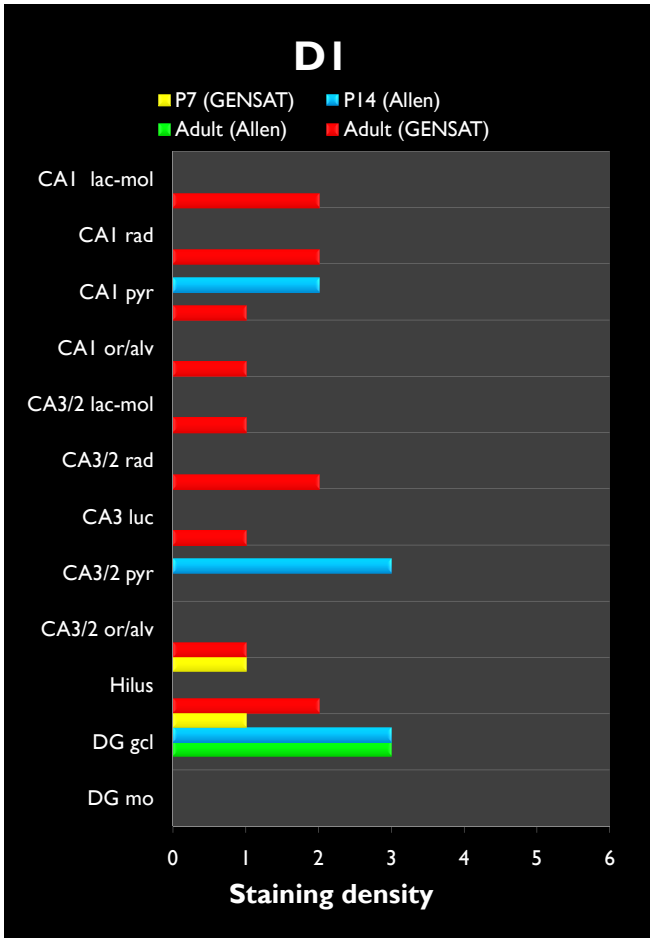
The results are illustrated and explained on the following pages, denoted by 5HT.





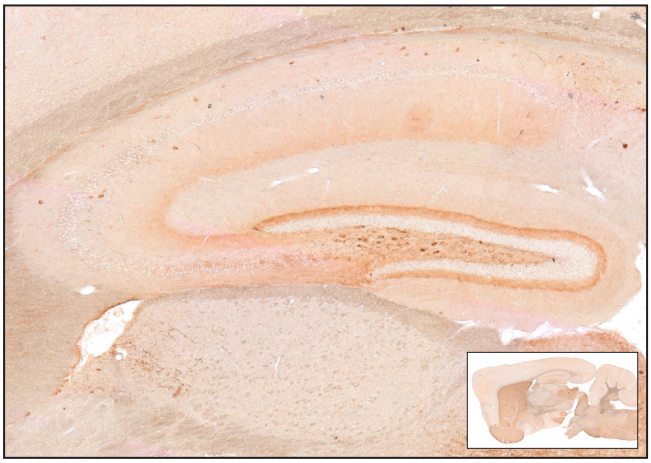


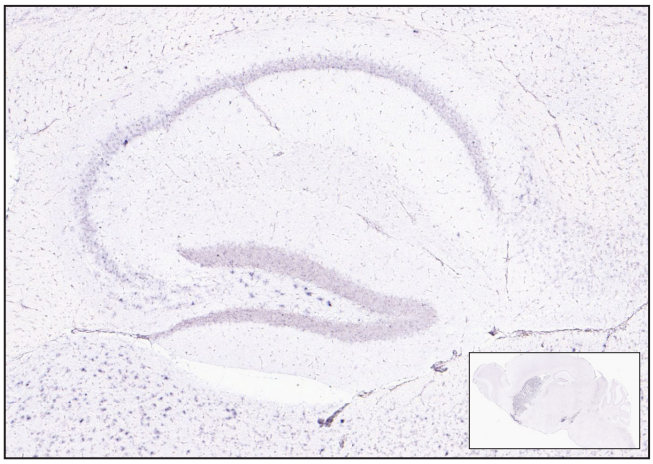


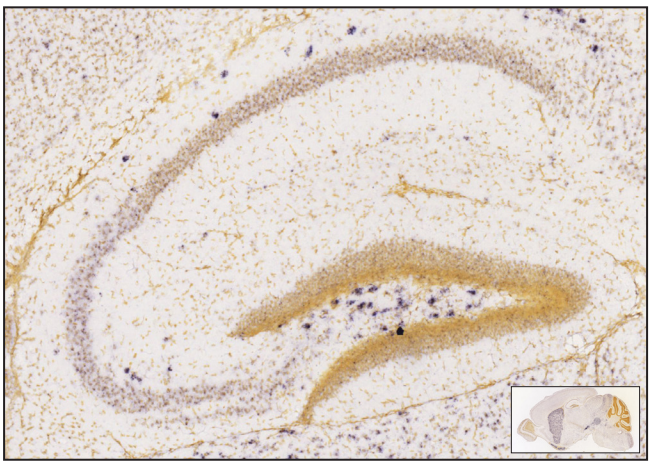


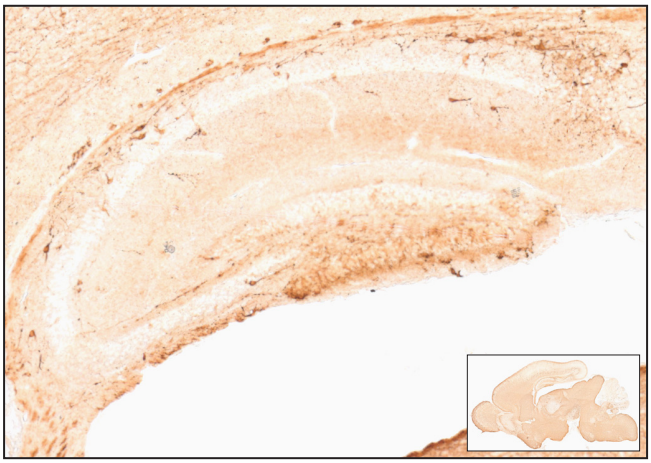
D1	Image series Id
Allen adult	71307280
Allen P14	100015333

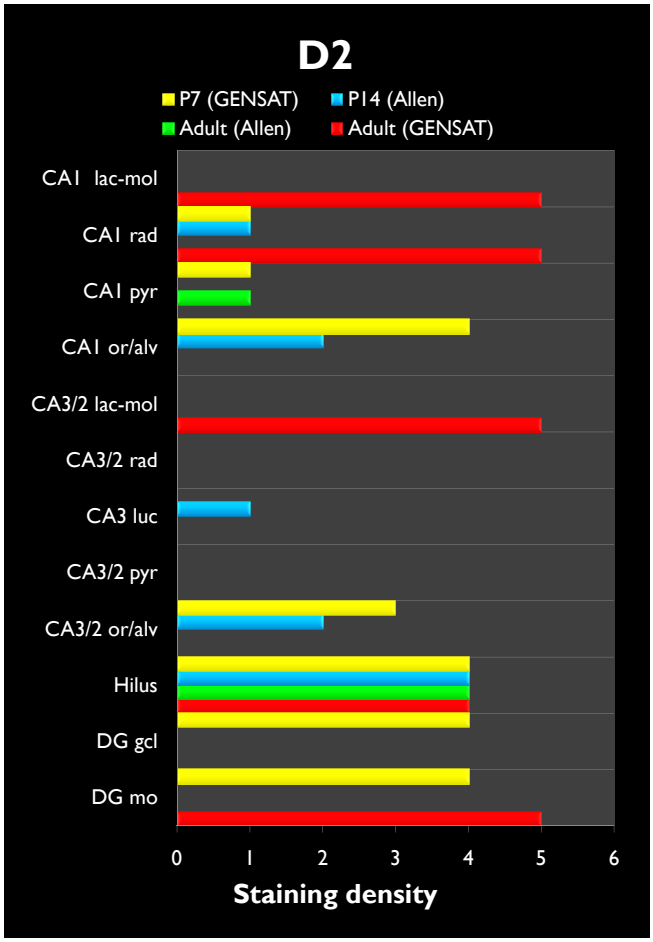
In the GENSAT adult mice there are scattered stained cells in the molecular layers of the entire hippocampus. Both the adult and the P14 animals are stained for mRNA in scattered cells of the dentate gyrus. In the P14 animals there are scattered cells stained in the pyramidal layers of CA3/2 and CA1 as well. The molecular layers display no staining. In the P7 animals single stained cells can be seen in the hilus and stratum oriens/alveus of CA3/2.





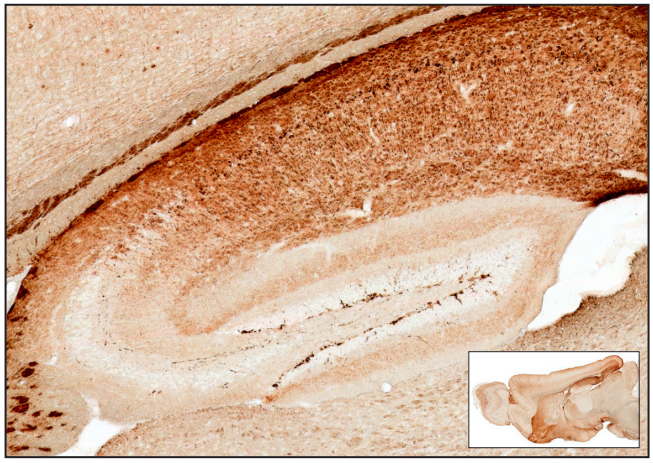


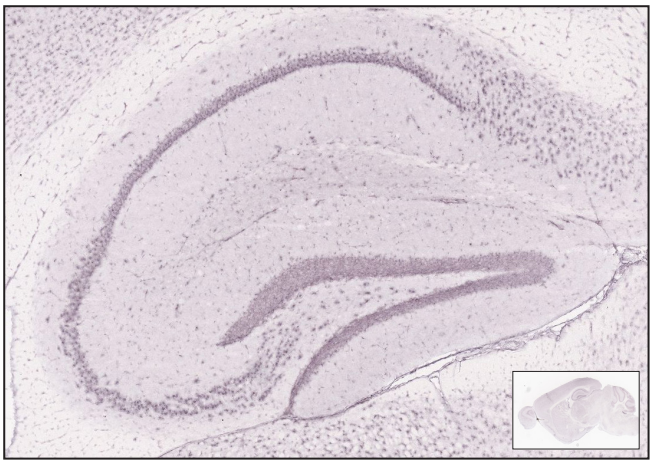


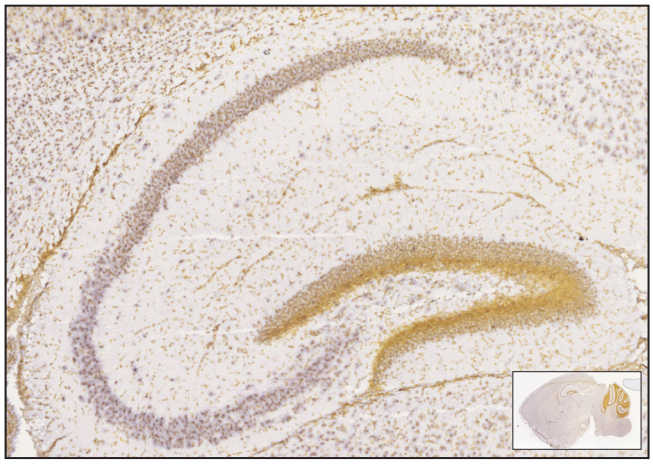


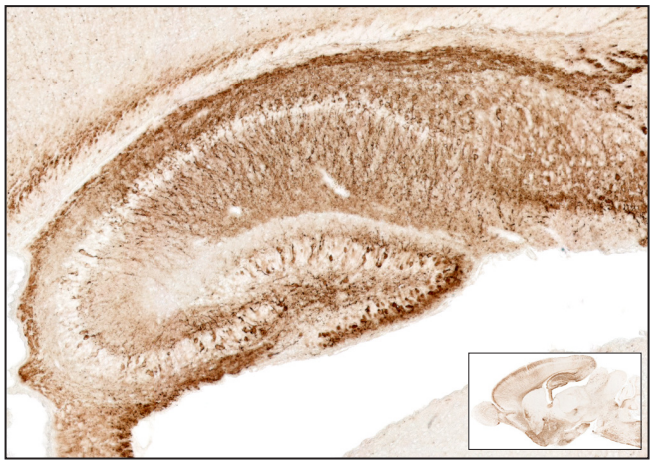
D2	Image series Id
Allen adult	81790728
Allen P14	100015332

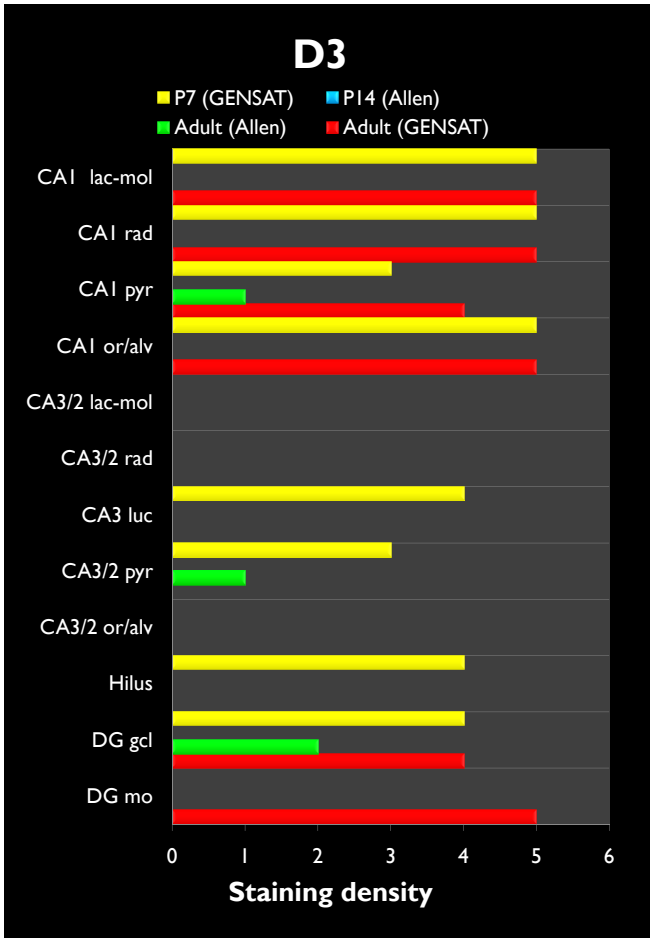
In the adult EGFP mice there is an ubiquitous laminar staining of several molecular layers. The inner molecular layer of the dentate, the lacunosum molecular of CA3/2, the lacunosum molecular of CA1, and a spread into the CA1 radiatum can be observed. Additionally scattered cell bodies in the hilus show staining. The scattered cell bodies can be seen in the Allen slides of both ages as well. Singly stained cells are seen in the CA1 pyramidal layer in the Allen adult slides. Scattered stained cells are present in the molecular layers of the Allen P14 mice. In the P7 the molecular layer of the ventral blade of the DG and the outer molecular layer of CA3/2 and CA1 show a strong stain. Consistent in all ages is the staining of scattered cell bodies in the hilus. In P7 the distribution is more widespread, but contained in parts of the molecular layer, specifically in the outer rim of the HC formation.





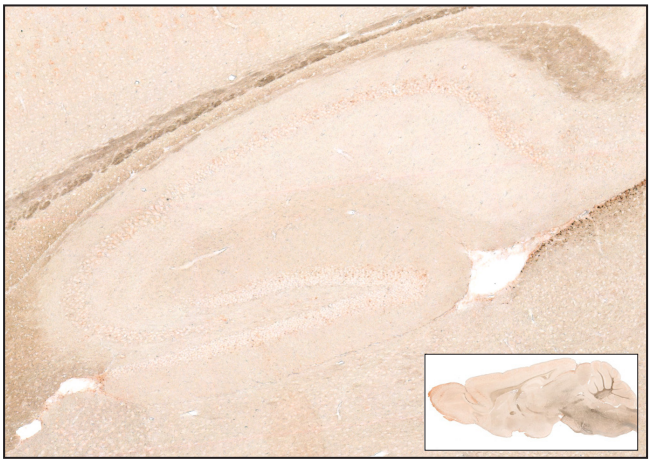


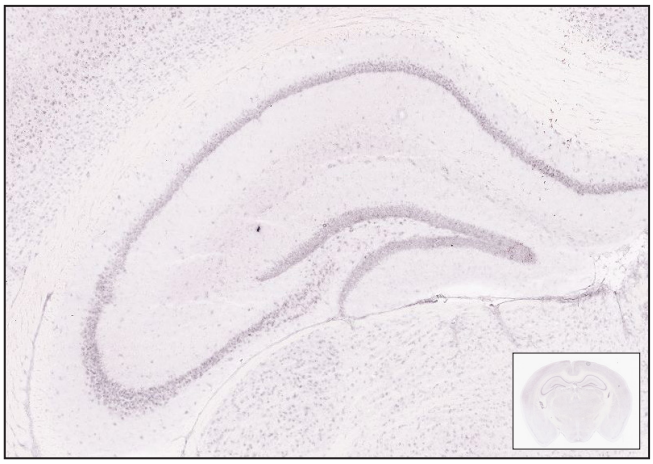


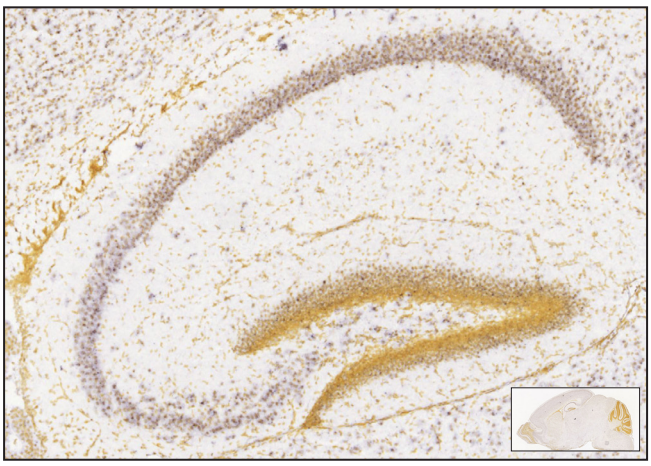


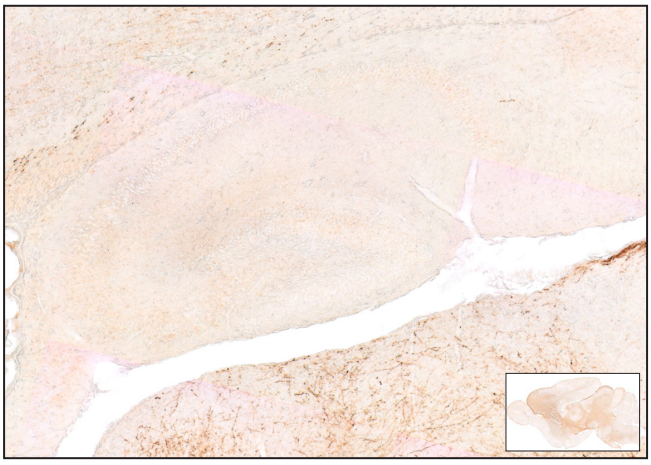
D3	Image series Id
Allen adult	100142530
Allen P14	100036963

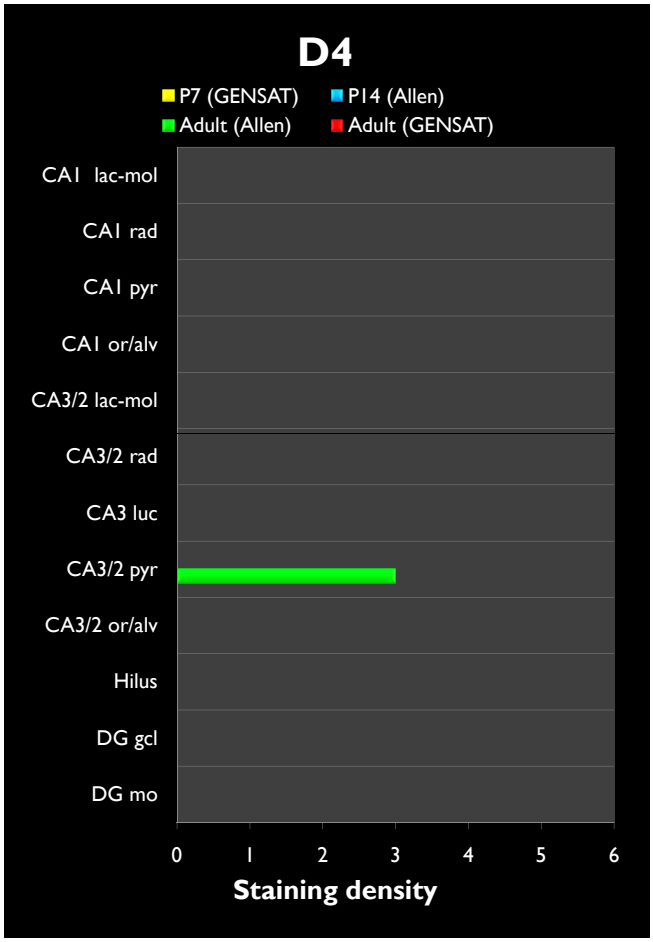
The D3 receptor expression show little consensus between the databases. In the Allen database the adult slides show a few scattered cells in the principal cell layers. In the GENSAT slides the distribution is dense, especially in the CA1 molecular layers. The staining pattern of the GENSAT adult and P7 are similar. Note however the lack of staining of the molecular layer in the P7 animals, and the present staining of the hilus stratum, lucidum of CA3 and the CA3/2 radiatum. Worth noting is the stained cells in the subgranular zone in both adult series. In the P7 slides the cells stained in the granule cell layer are not confined to the subgranular zone but scattered in the entire GCL.





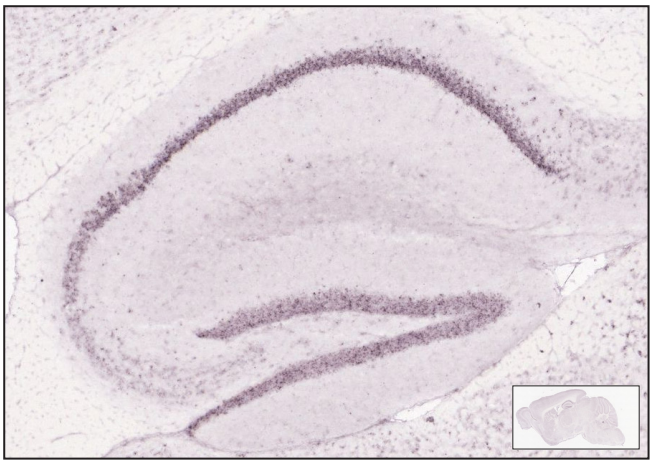


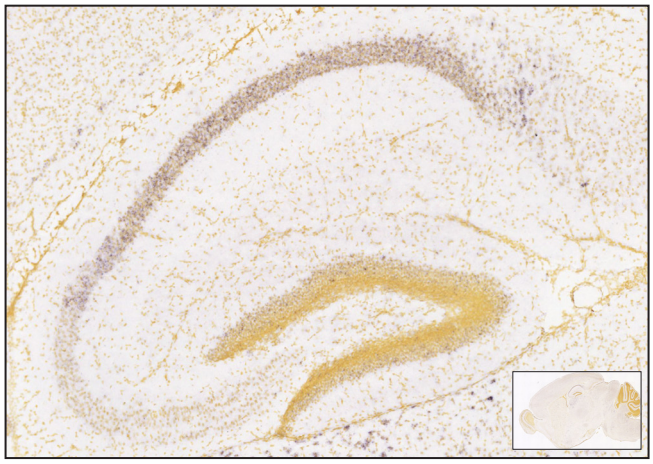


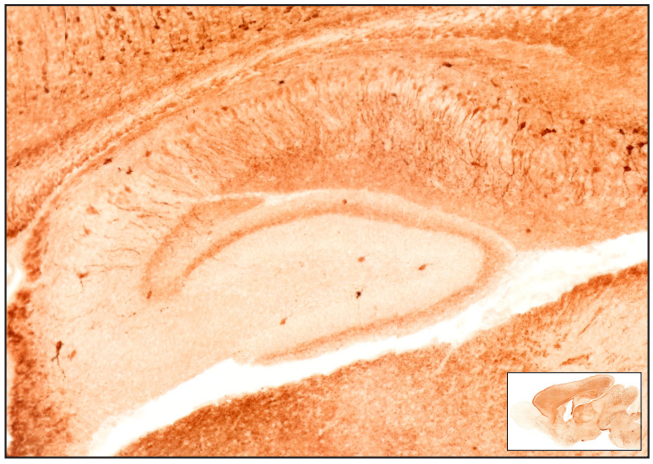


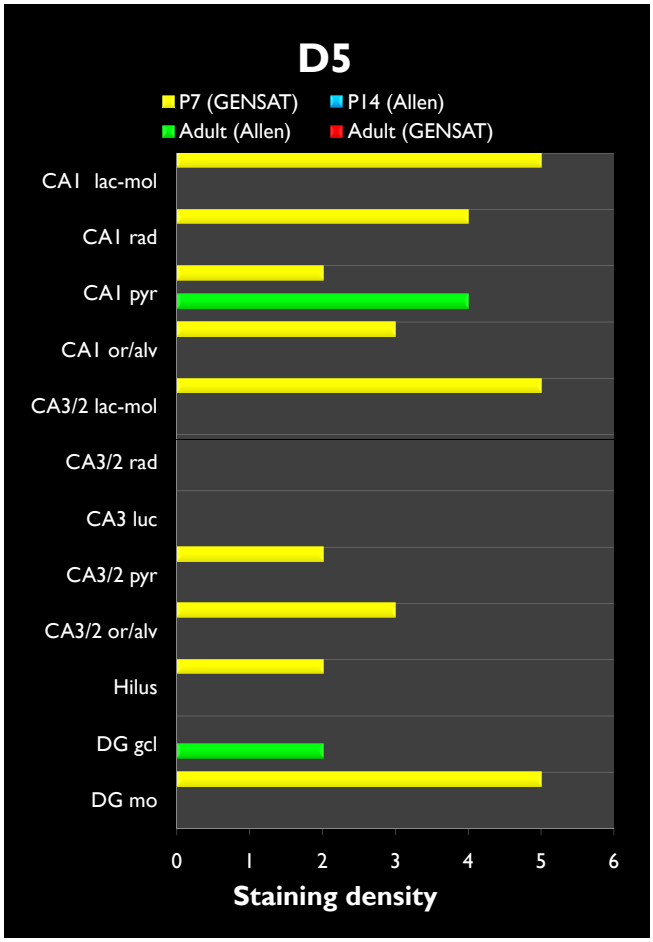
D4	Image series Id
Allen adult	112650336
Allen P14	100075324

Only the Allen adult series show a few scattered cells in the pyramidal layer of CA3/2. In the others there are no discernible staining in the hippocampus.



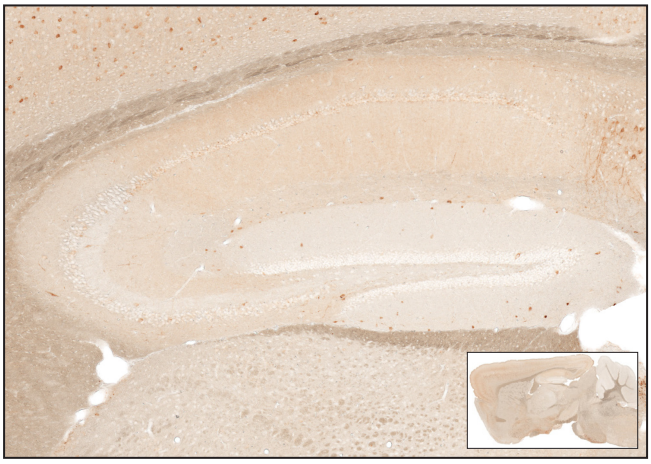


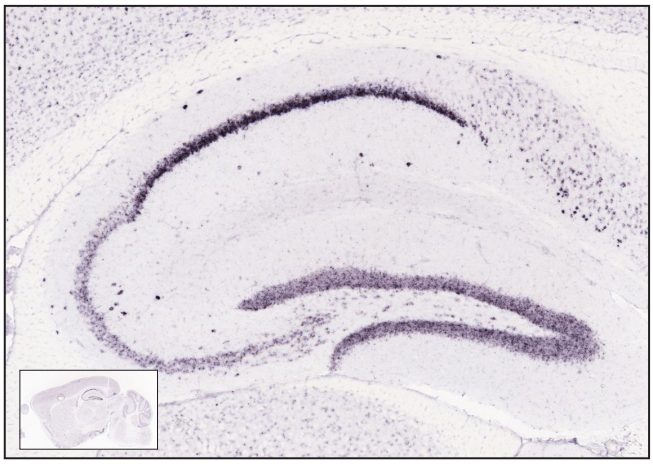


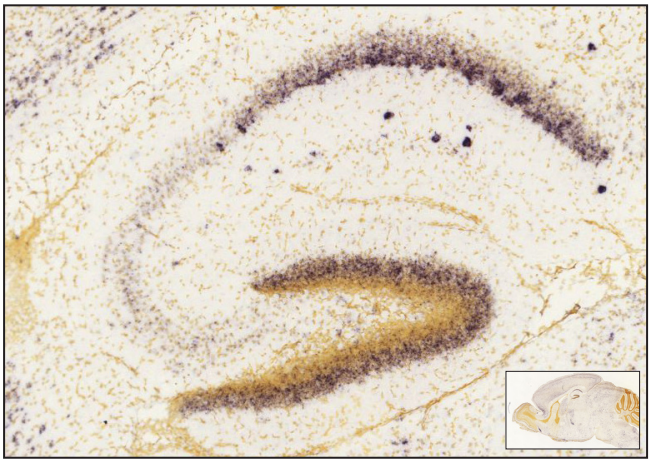


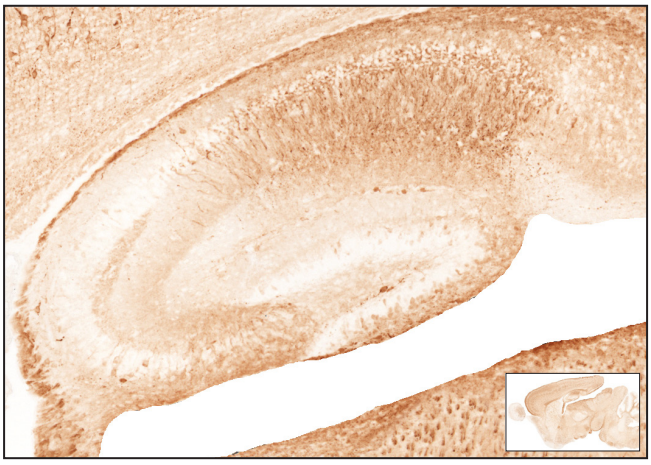
D5	Image series Id
Allen adult	81790710
Allen P14	100093944

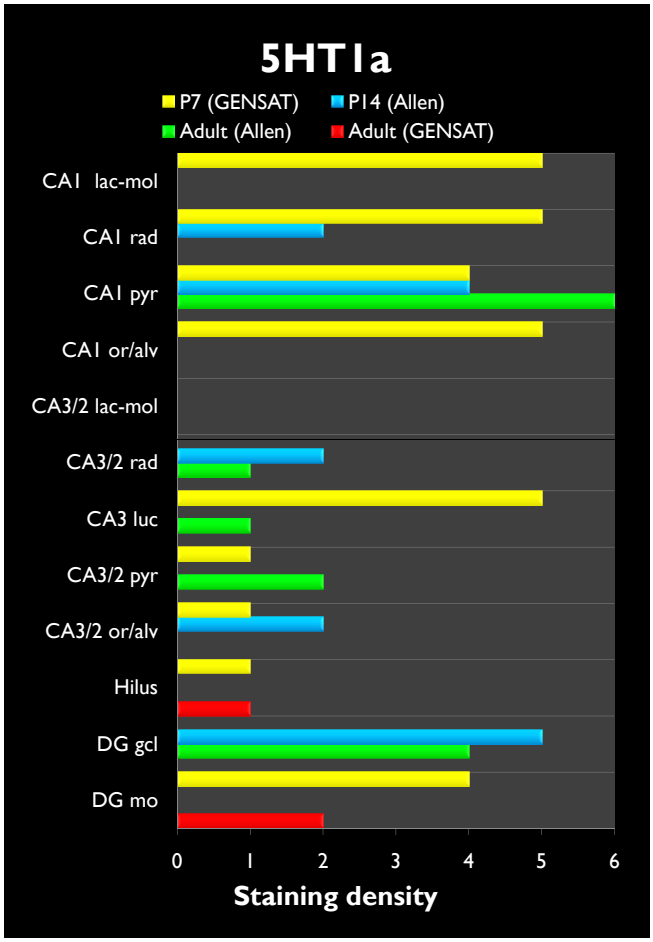
The D5 receptor has a dense staining pattern in the CA1 of the Allen adult slides, and scattered cells in the dentate gyrus. Interestingly both the GENSAT adult and the Allen P14 show no expression. A change in the expression pattern can be seen in the P7 animals, notably there is a layered ubiquitous staining in the DG molecular layer. The CA molecular layers are stained with scattered cells in the pyramidal layers. There is a gradual increase in staining intensity as one moves through the trisynaptic pathway, increasing from no staining of the DG GCL and hilus to a stain that increases in intensity in CA3 as it progress towards CA1.





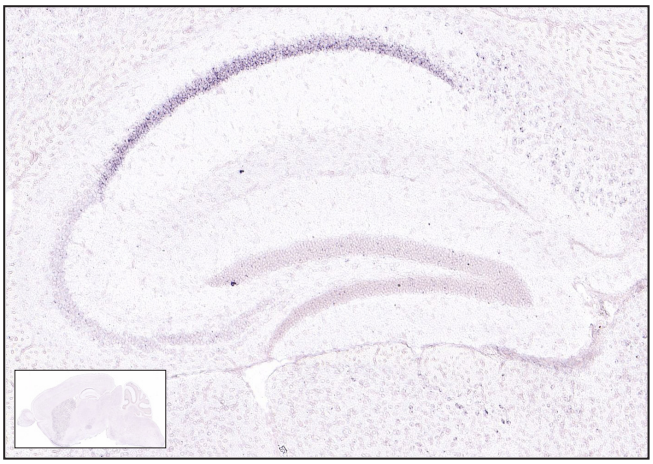


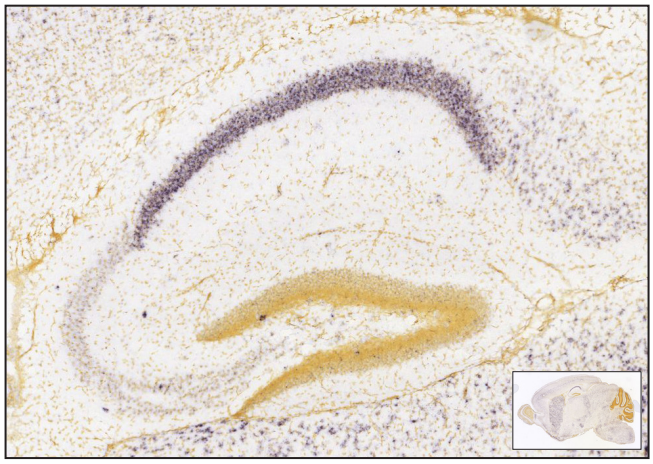


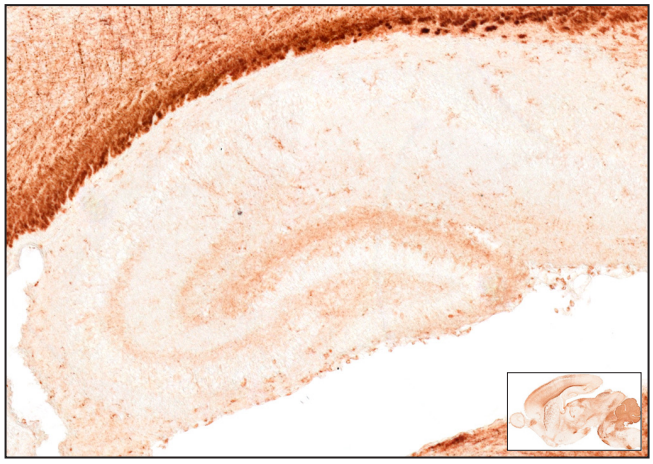


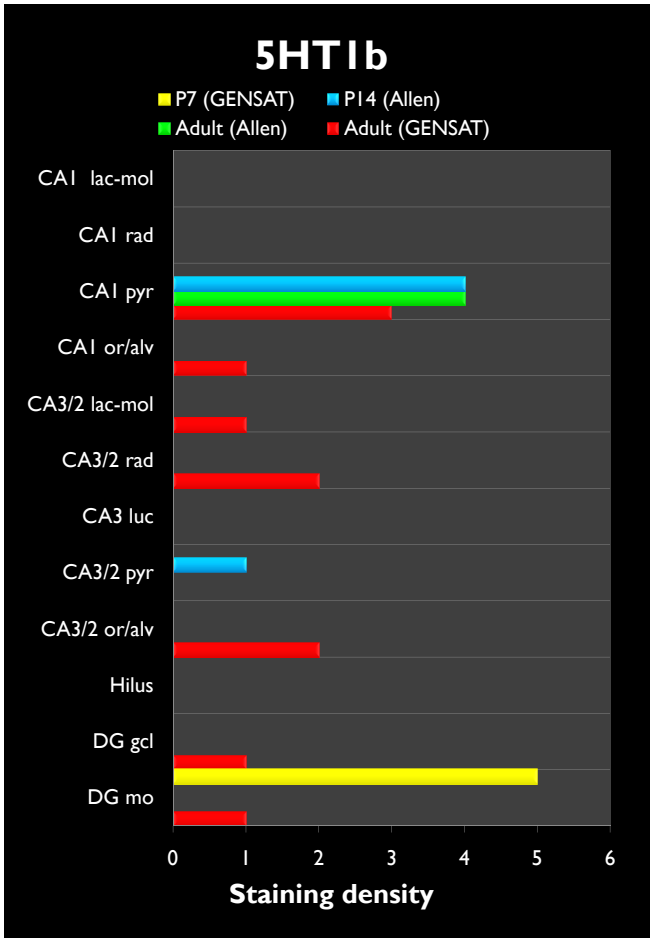
5HT1a	Image series Id
Allen adult	79394355
Allen P14	100015325

5ht1a show a few scattered stained cells in the hilus and dentate gyrus molecular layer in the GENSAT adult series. In the Allen adult series expression is primarily found in the CA1 pyramidal layer and dentate gyrus granule cell layer. There is some indication of a spatially ordered distribution, where the highest density can be seen in the basal CA1 pyramidal layer. Scattered cells can also be seen in the molecular layers and pyramidal layer of CA3/2. In the GENSAT P7 series there is a similar staining to both Allen series. Additionally the hilar mossy fiber projections from the DG to CA3 stratum lucidum are densely stained, the molecular layer of the dentate ventral blade is stained, and the molecular layers of the CA1 are densely stained. Worth noting is the layer specific dense staining of CA3/2 stratum radiatum.



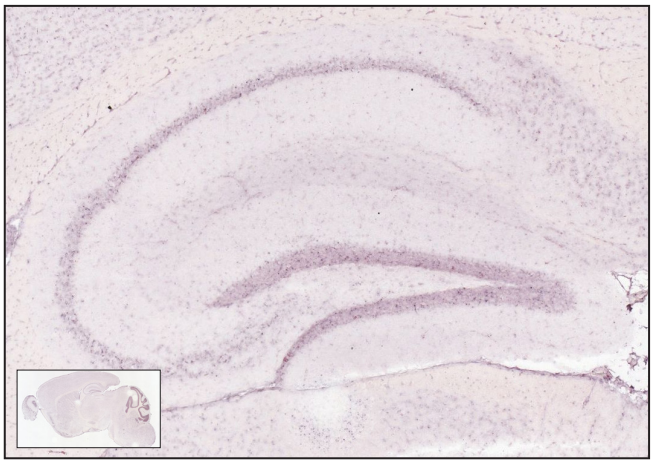


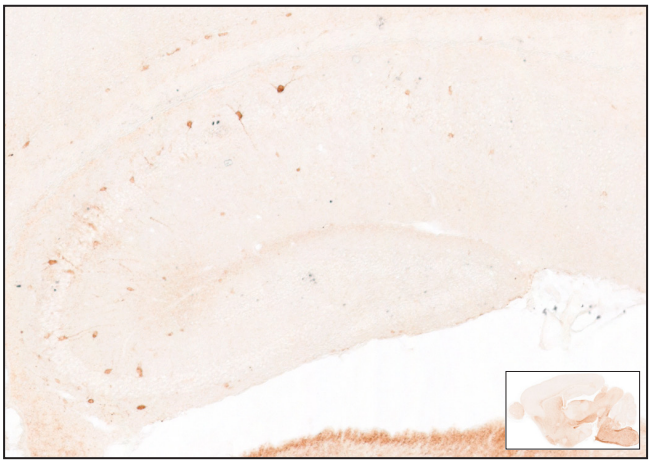


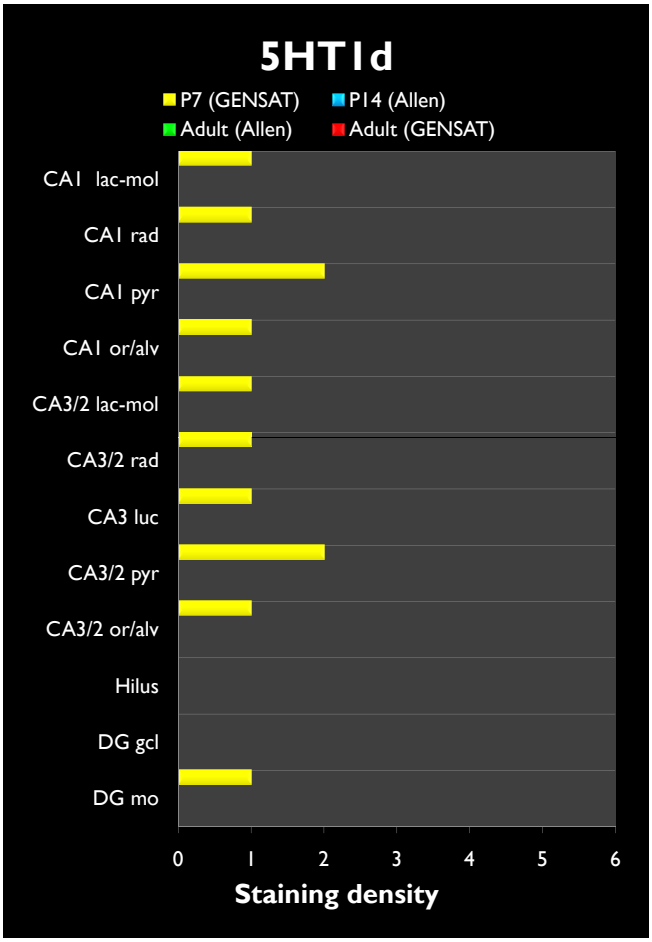


5HT1b	Image series Id
Allen adult	79913318
Allen P14	100015326

The GENSAT adult show scattered stained cell bodies in various molecular and principal cell layer. A notable increase in density is seen in the CA1 pyramidal layer. In the CA1 pyramidal layer of the Allen adult series the staining is denser than in the GENSAT adult series. The P14 confirm this staining pattern. A ubiquitous layered staining of the P7 outer DG molecular layer can be seen. The staining intensity of the DG molecular layer show great variance between series.



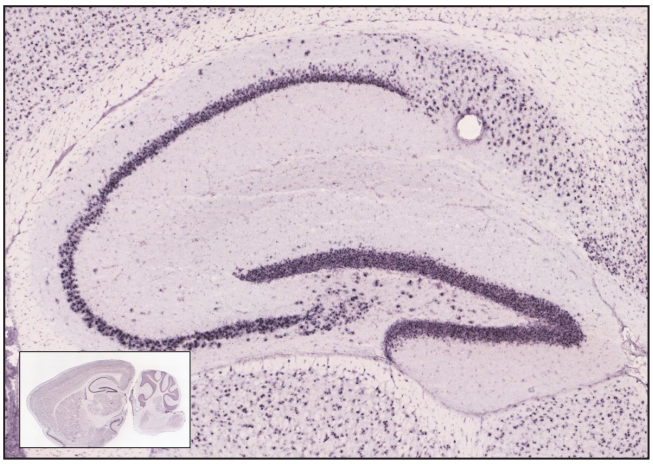


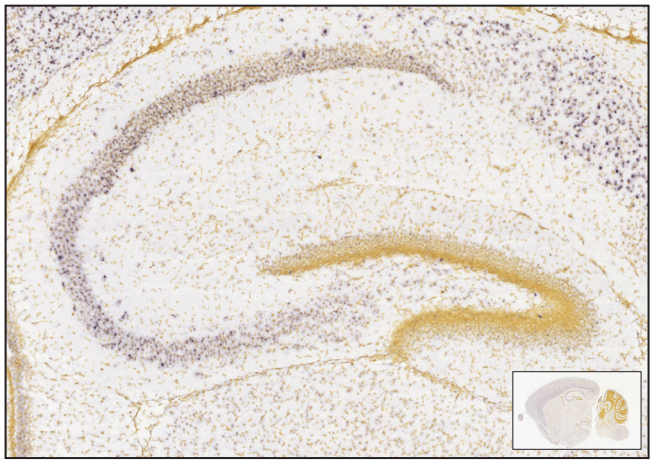


5HT1d	Image series Id
Allen adult	71393418
Allen P14	100083194

Only the GENSAT P7 mice show any staining of the 5HT1d. The staining are single and scattered cell bodies spread out across all areas except the hilus and granule cell layer.

NO DATA





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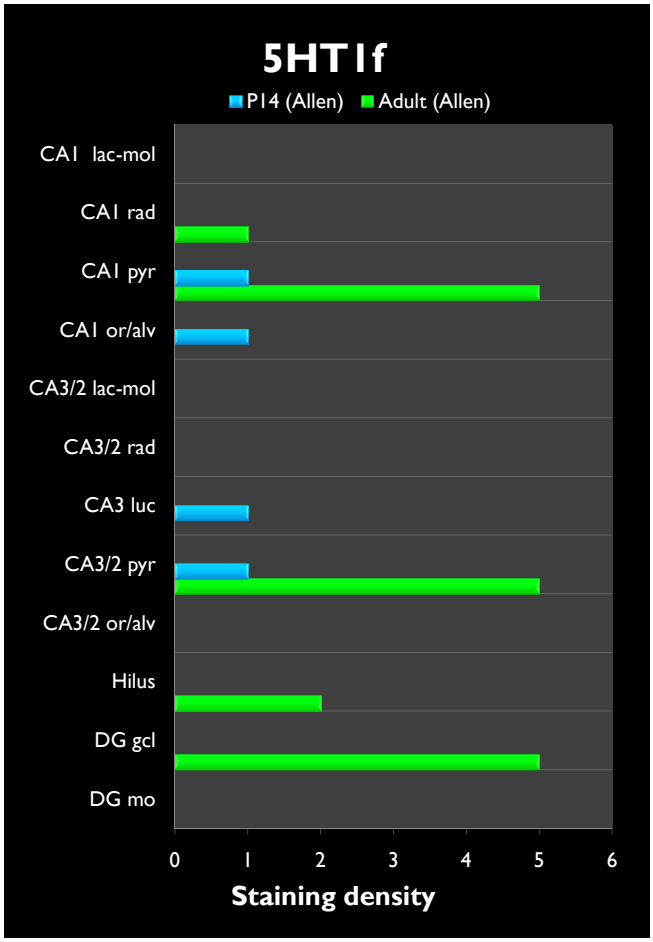
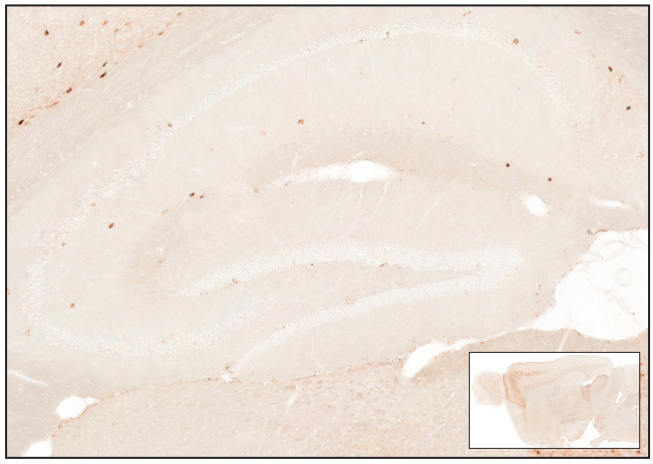
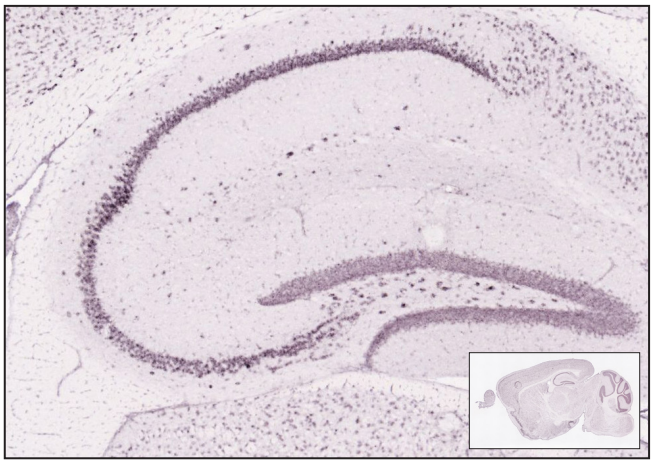
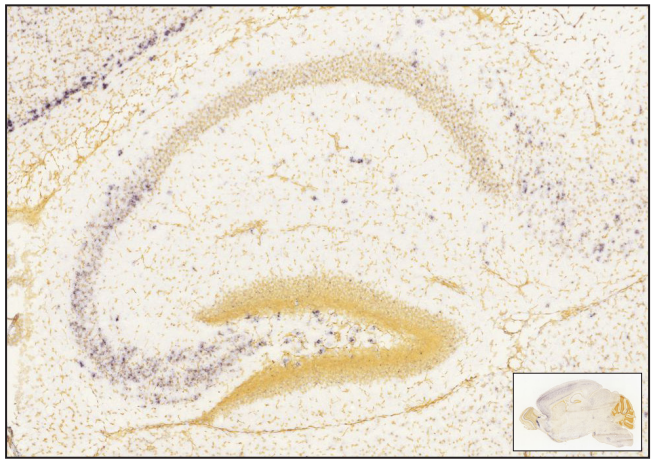


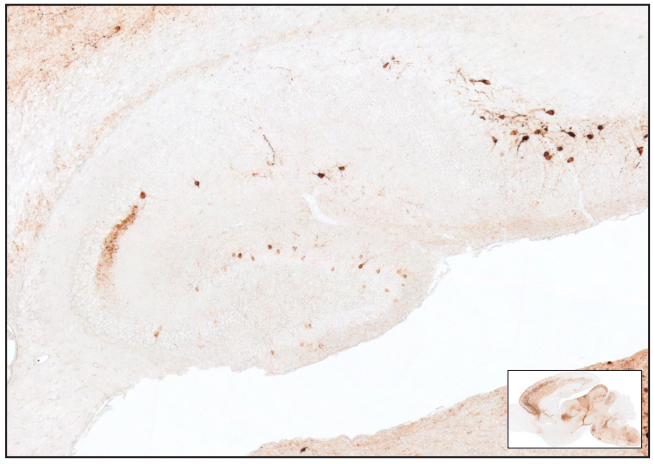
5HT1f	Image series Id
Allen adult	69859867
Allen P14	100070328

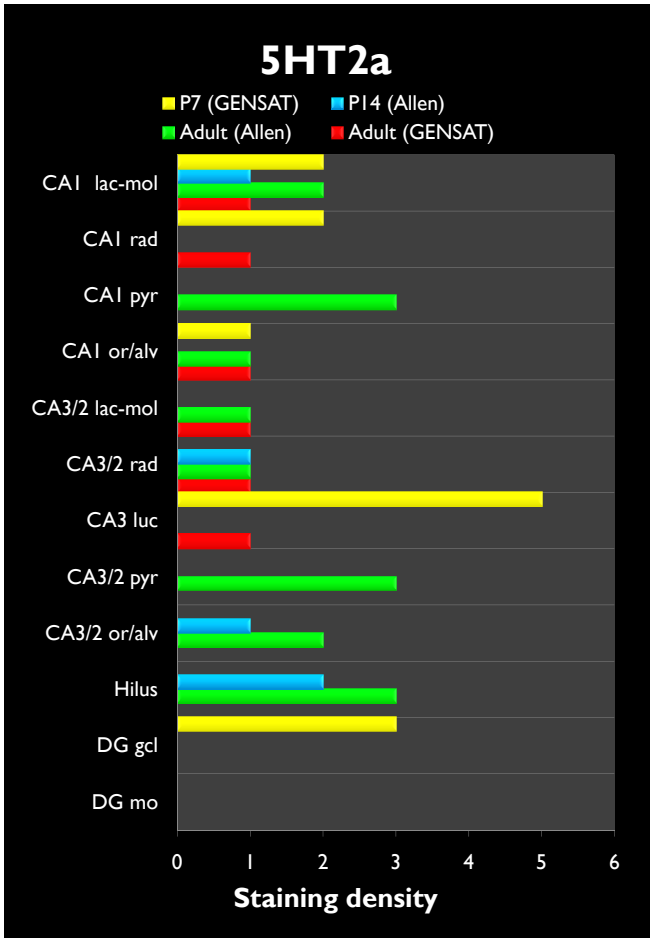
The gene for the 5HT1f receptor is too large for BAC transgenesis due to its 181kb length, and is not represented in the GENSAT database. In the Allen adult series the principal layers of the HC are all densely stained. Additionally scattered stained cell bodies in the hilus and stratum radiatum are present. This staining pattern is not present in the P14 slice, and there are only singly stained cell bodies in the CA pyramidal layers and the CA3 lucidum and CA1 alveus/oriens.







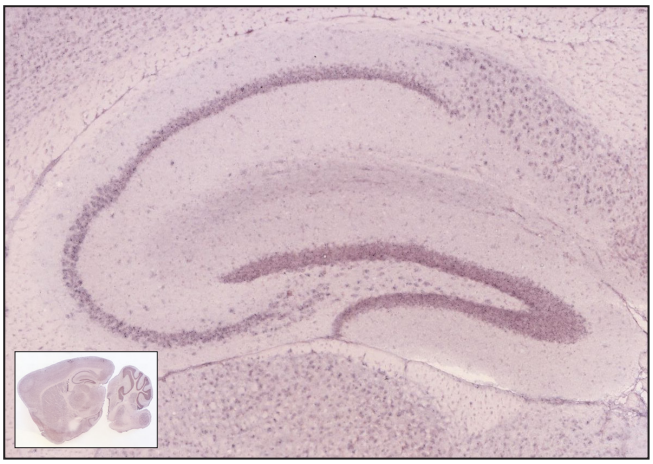


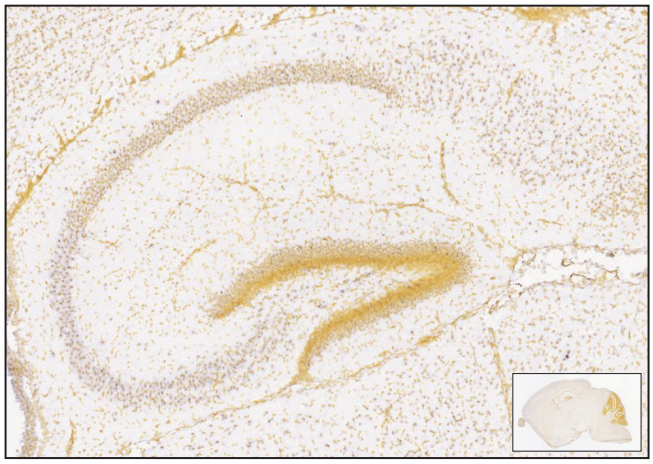


5HT2a	Image series Id
Allen adult	81671344
Allen P14	100015327

In the GENSAT adult slides a few scattered single cells in the molecular layers of CA1-3 and is seen. A similar pattern is seen in the Allen adult series with a notable addition of a medium dense staining of the pyramidal layers and scattered cell bodies in the hilus. The Allen P14 series does not show staining of the pyramidal layers, but mirror the hilar staining pattern. This series also show single cell body staining in CA molecular layers. The GENSAT P7 series show scatted cell bodies in the CA1 molecular layers. A notable feature of this series is the ubiquitous staining of parts of the CA3 stratum lucidum, and the medium-dense staining of the dentae gyrus granule cell layer.

NO DATA





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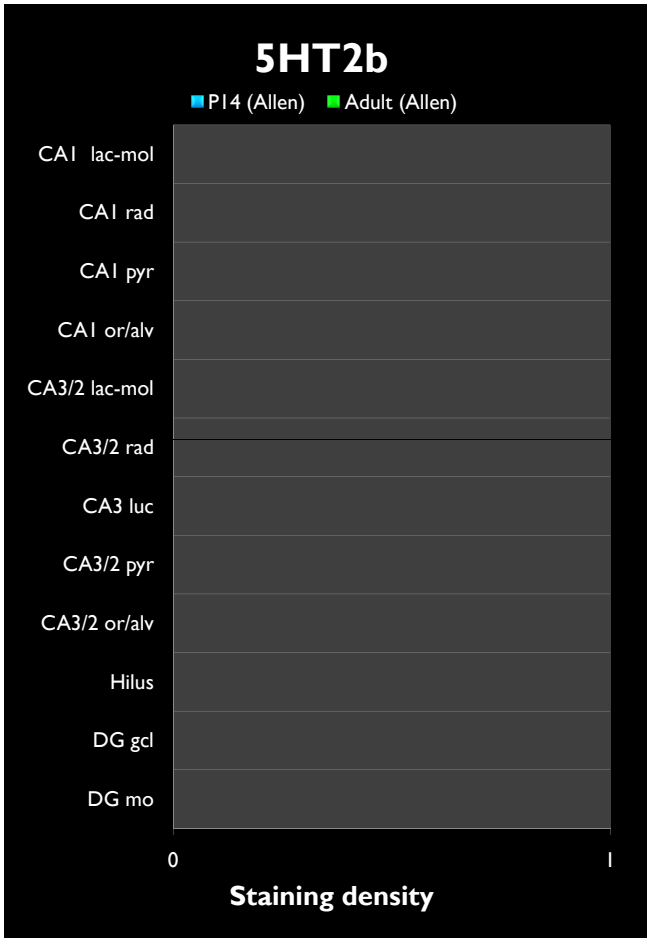
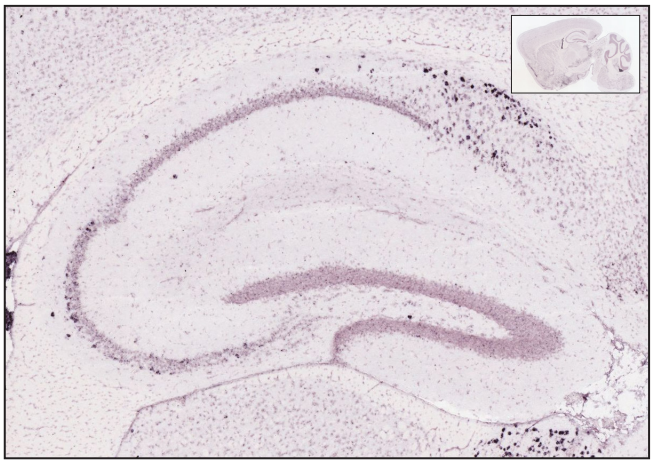
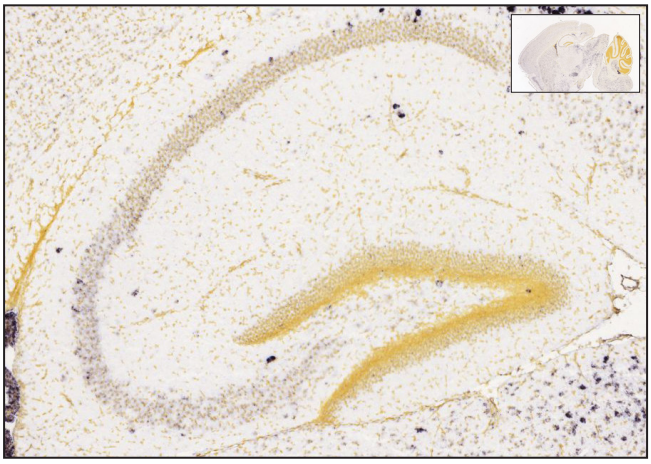


5HT2b	Image series Id
Allen adult	71664130
Allen P14	100039420

The GENSAT database have not produced transgenic mice for this gene, and BAC targeting is no longer ongoing. No discernible staining of the HC formation in any of the Allen series.

NO DATA





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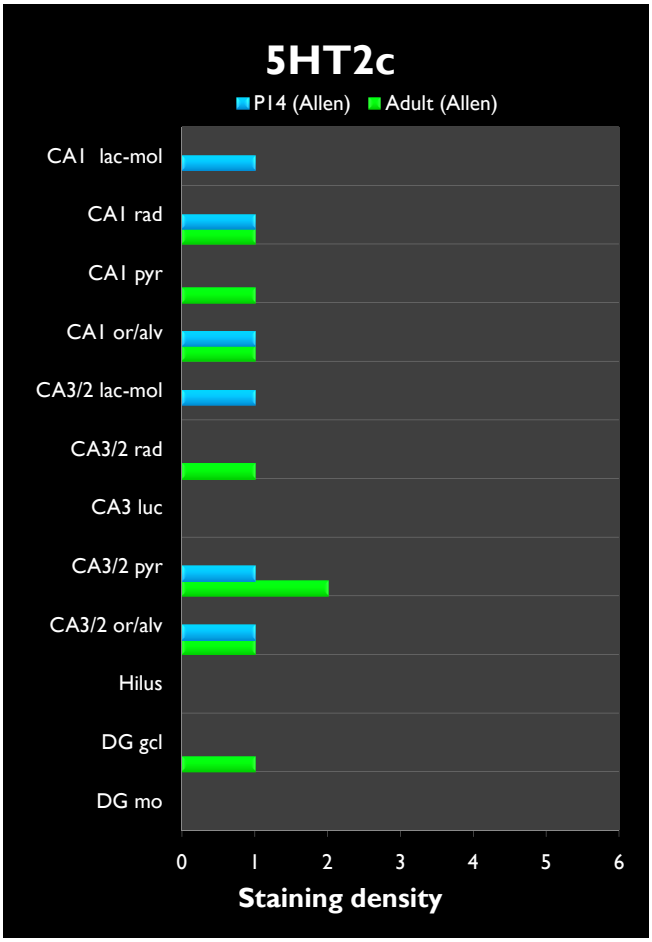
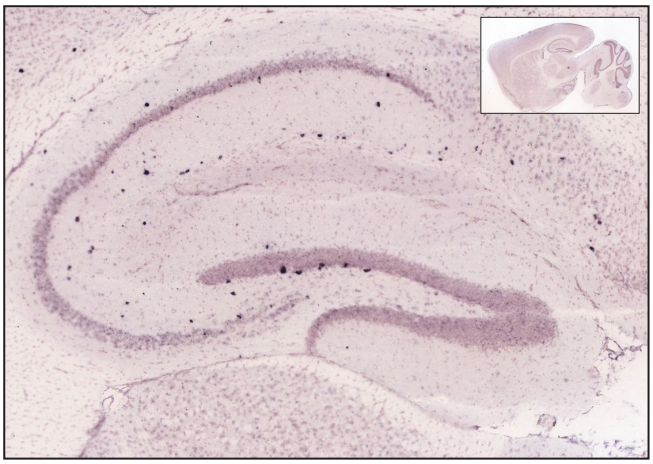
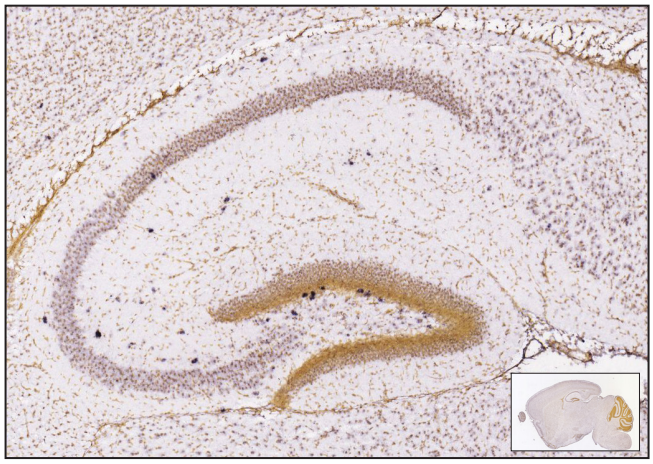
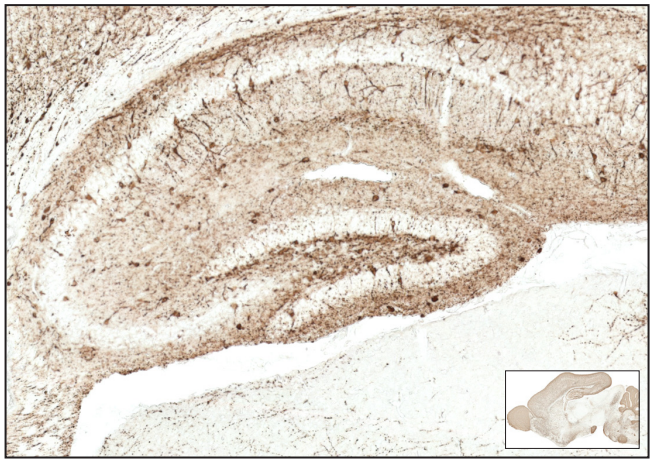


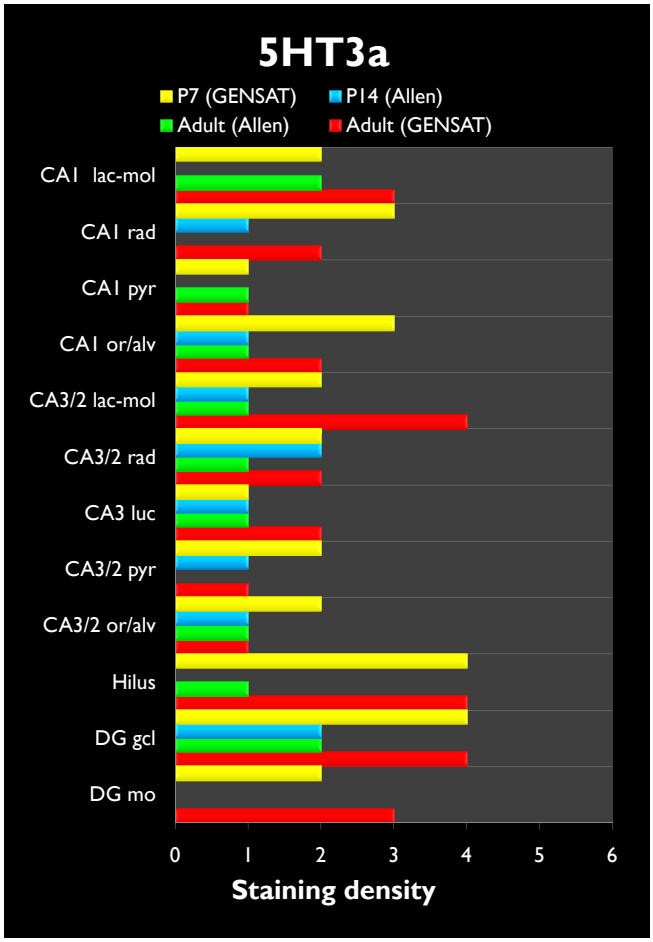
5HT2c	Image series Id
Allen adult	71393424
Allen P14	100045428

The gene for the 5HT2c receptor is too large for BAC transgenesis due to its 235kb length, and is not represented in the GENSAT database. In the Allen adult series there a singly expressing cell bodies in the principal cell layers, and some of the molecular layers. A denser distribution is seen in the CA3/2 pyramidal layer. The staining pattern is similar in the Allen P14 series, but present in fewer areas.



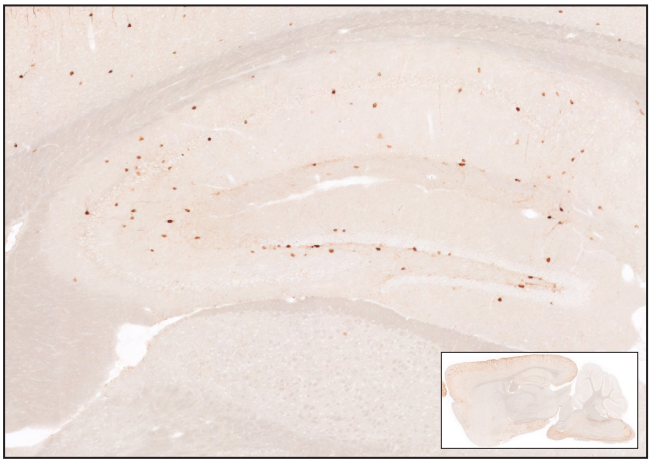


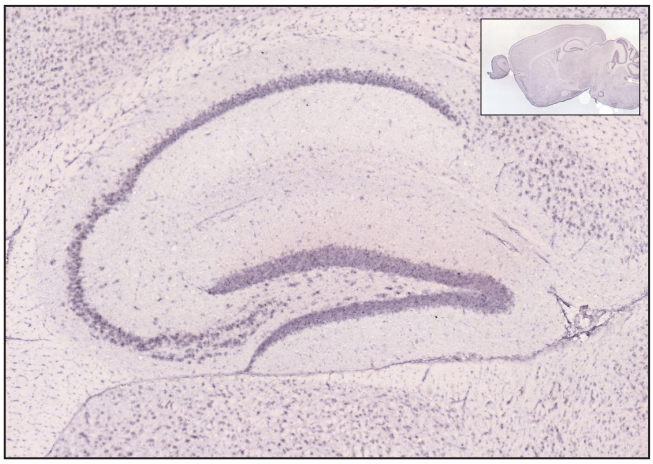


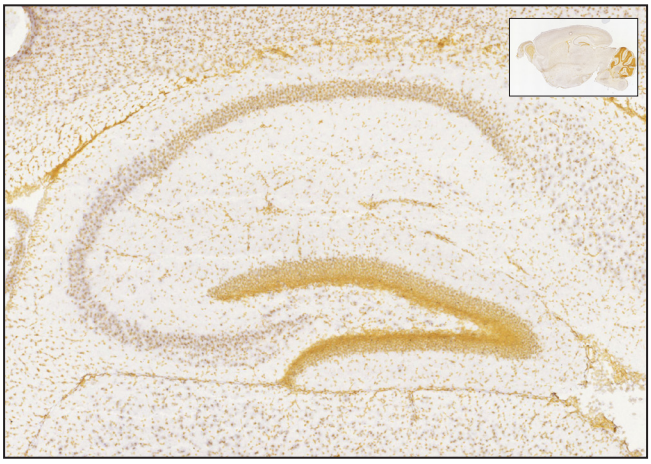


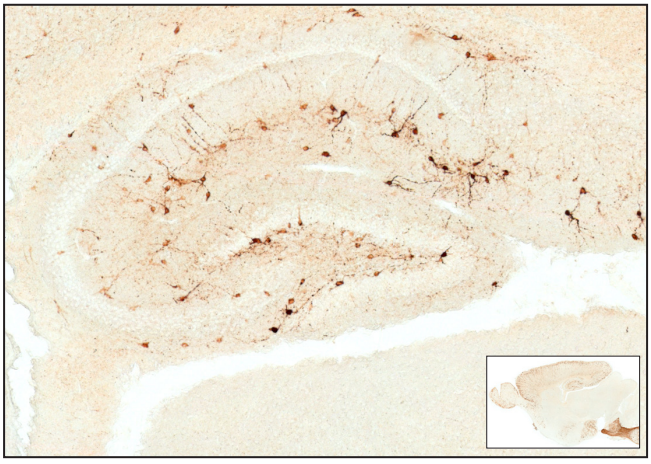
5HT3a	Image series Id
Allen adult	70593142
Allen P14	100041454

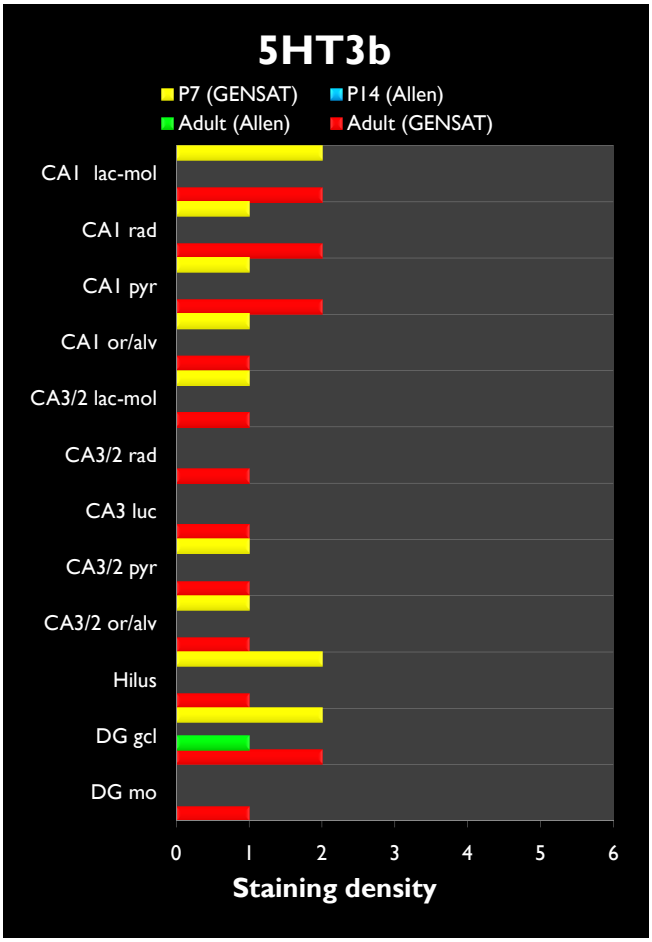
In the GENSAT adult series the receptor is expressed in scattered cells in the molecular layers and pyramidal layers of the cornu ammonis. The expression is most dense in the CA3/2and CA1 lacunosum moleculare, the hilus, basal area of the dentate gyrus granular cell layer and thedentate gyrus molecular layer. In the Allen adult series, the singly satined cell bodies are present in most layers, with increased density in the basal granular cell layer of the dentate gyrus and the CA1 lacunosum molecular. The distribution is similar in the P14 animals. The GENSAT P7 series show similar expression to that of the adult. Note the concentration of the staining of the DG molecular in the ventral blade. In both series the greatest density can be seen in the hilus.





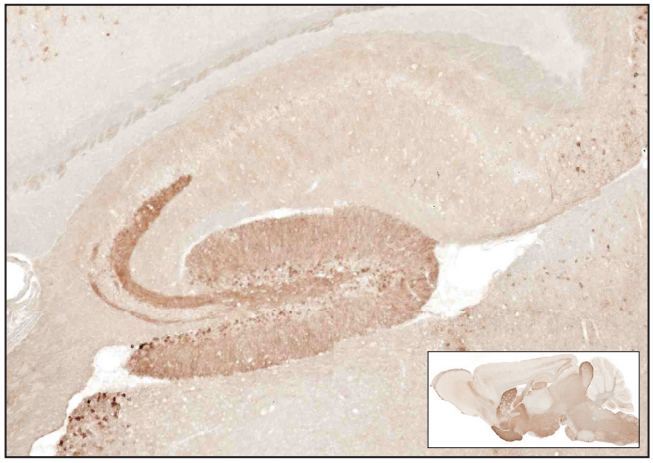


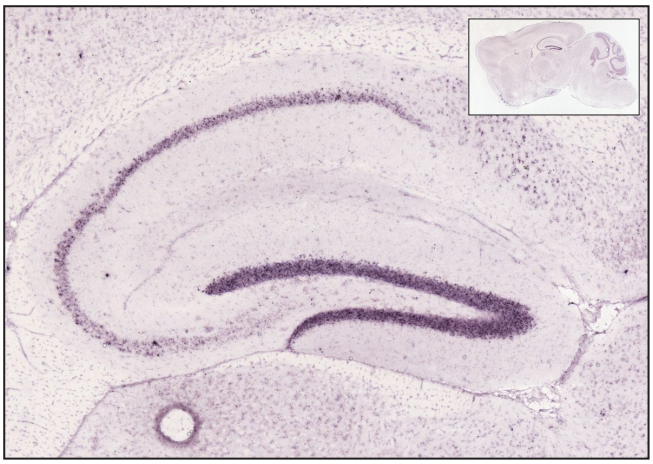


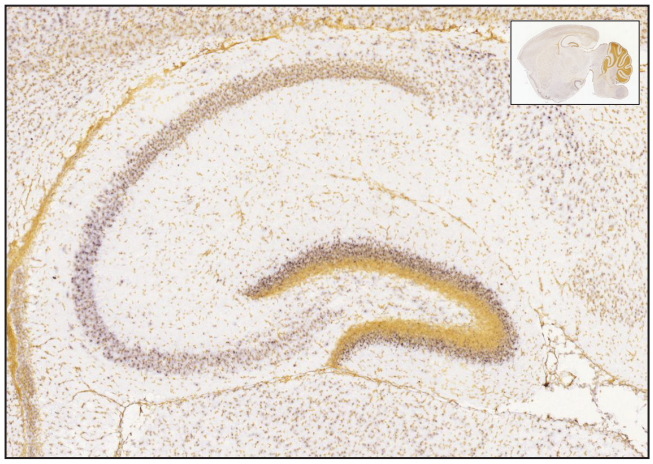


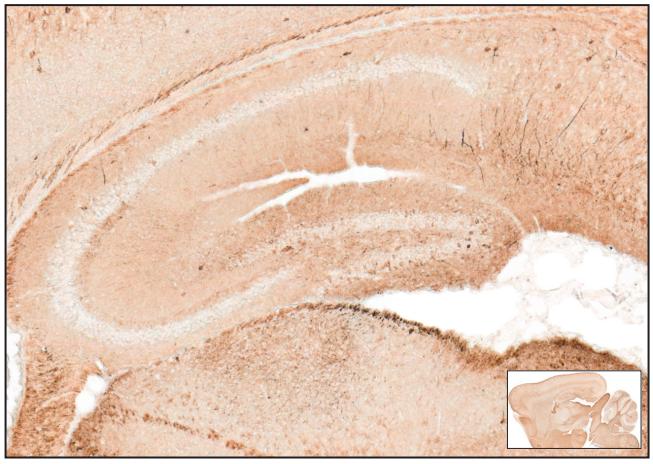
5HT3b	Image series Id
Allen adult	68745408
Allen P14	100041007

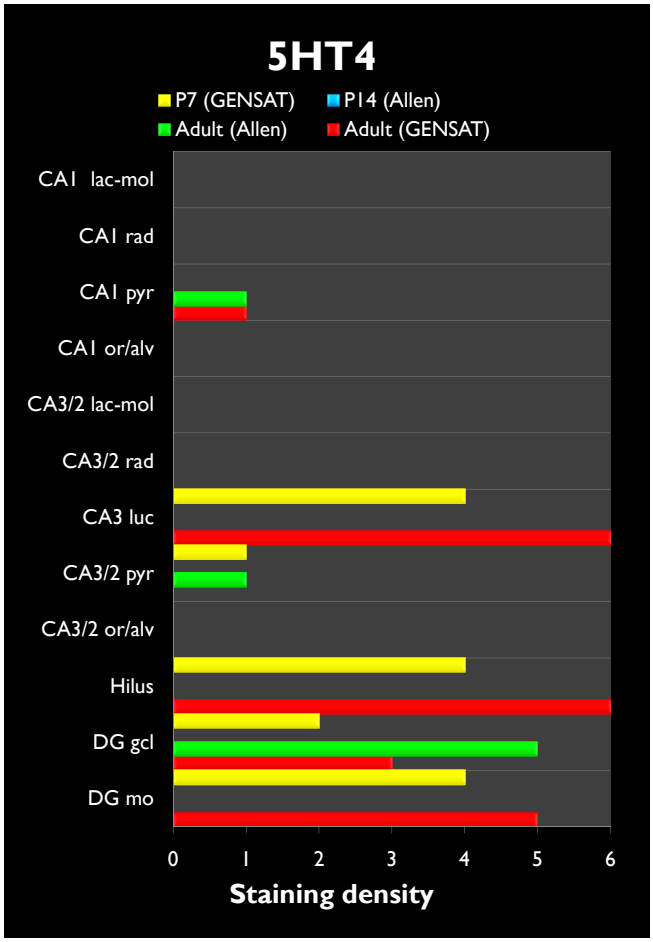
In the adult GENSAT series there is scattered cell across all hippocampal layers. Increased densities are seen in CA1 and the dentate gyrus basal granule cell layer. In the Allen adult series only singly stained cell bodies are present. In the Allen P14 series there is no discernible staining of the hippocampus. The GENSAT P7 animals a similar staining to the adult series. Note that the staining is more layered in the adult, and less present in the CA3.





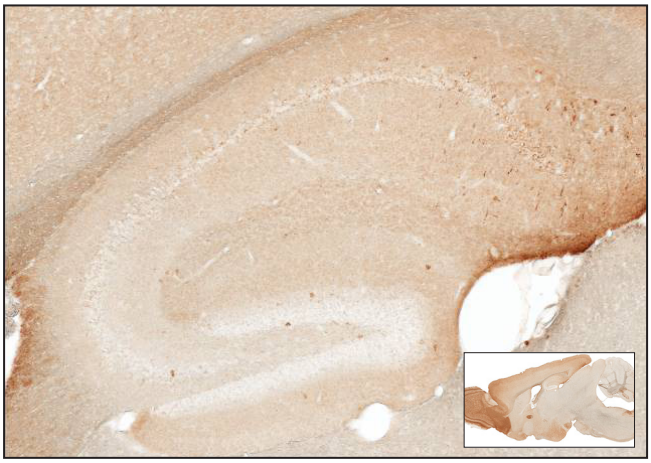


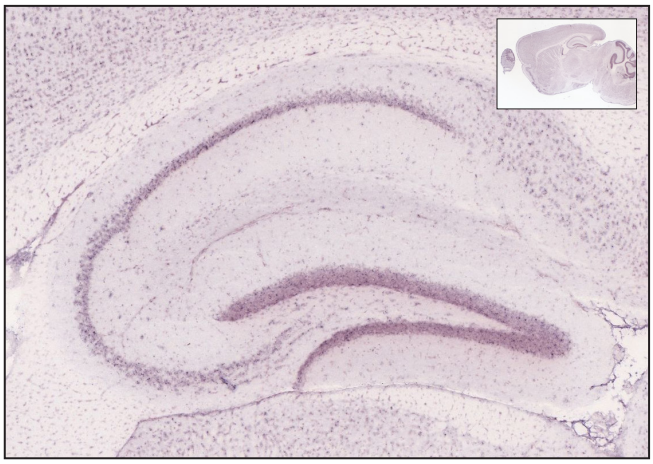


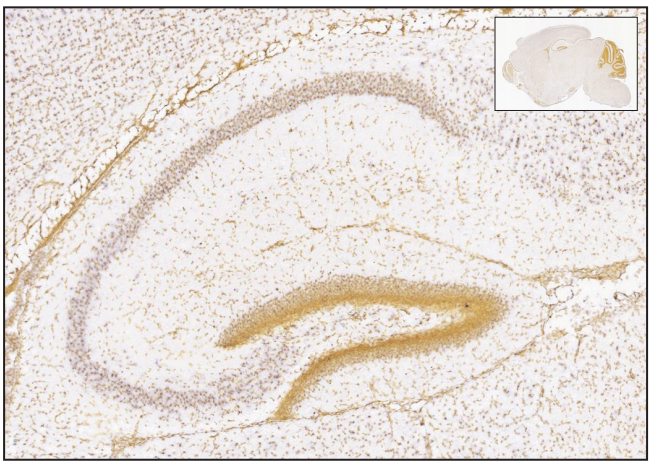


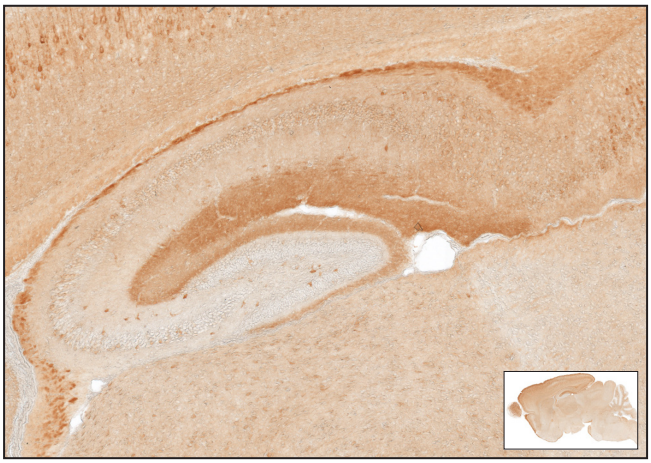
5HT4	Image series Id
Allen adult	69257849
Allen P14	100041463

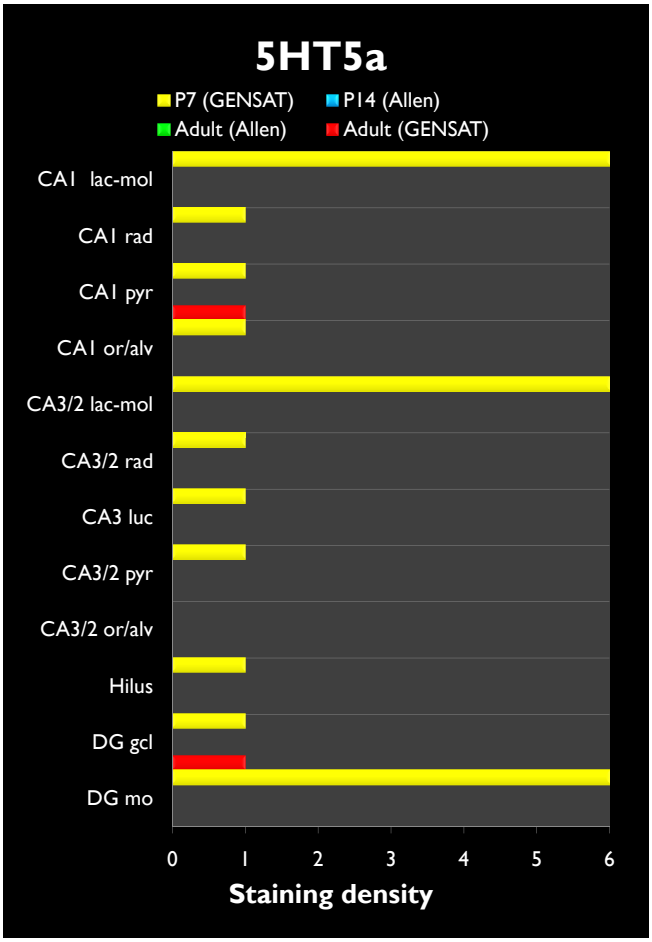
In the GENSAT adult series the dentate gyrus molecular layer and hilus are ubiquitously stained. In the granule cell layer scattered stained cell bodies can be seen. The stratum lucidum of the CA3 show the same ubiquitous staining as the hilus, the rest of cornu ammonis are unstained. In the Allen adult slides the granule cell layer is densely stained, with singly stained cell bodies spread in the pyramidal layers. The Allen P14 series has no discernible staining. In the GENSAT P7 series the distribution is similar to the adult series, but with a generally decreased density. Note that the P7 series also show singly stained cell bodies in the CA3/2 pyramidal layer.





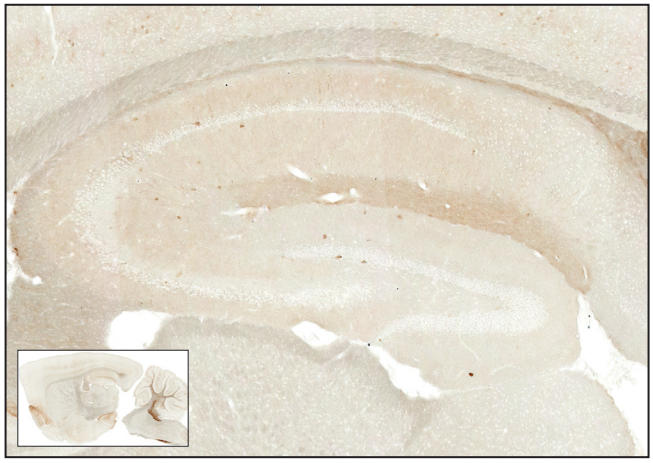


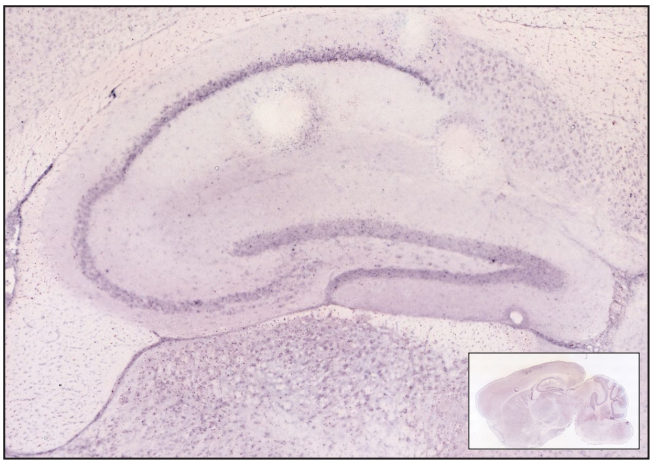


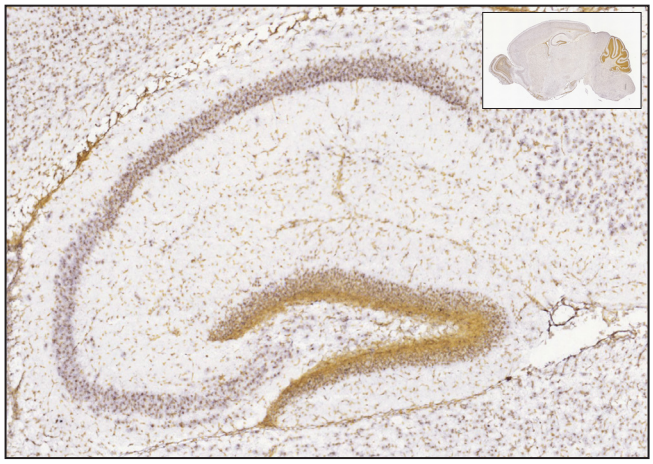


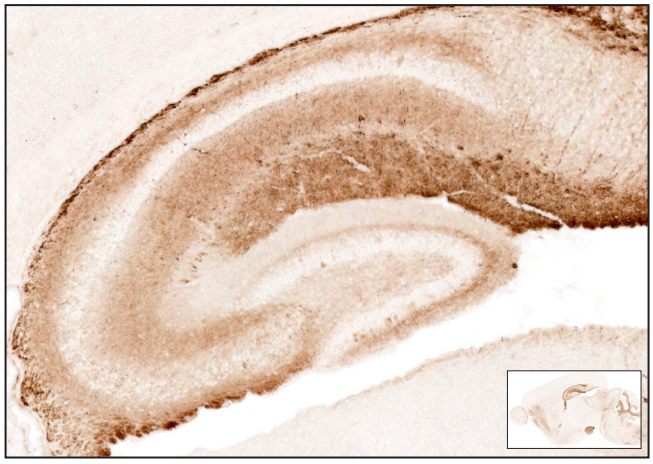
5HT5a	Image series Id
Allen adult	71393430
Allen P14	100071940

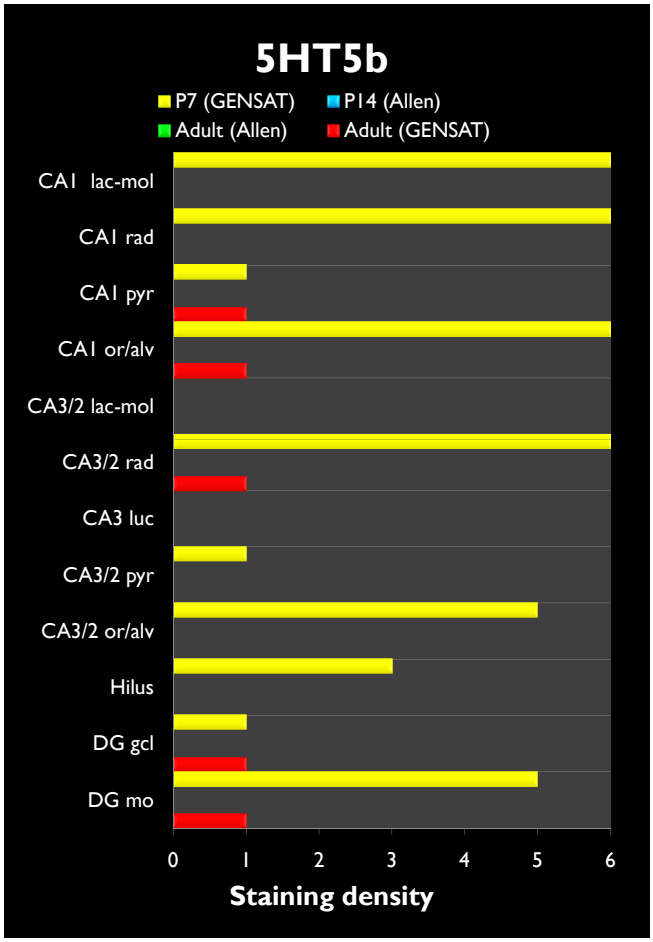
In the GENSAT adult there are few singly stained cell bodies in the dentate gyrus granule cell layer and the CA1 pyramidal layer. Apart from that the series is unstained. Both the Allen adult and the Allen P14 show no staining. Interestingly the GENSAT P7 series show a markedly different staining pattern. Note in particular the layered ubiquitous staining in the outer dentate gyrus molecular layer and the stratum lacunosum moleculare of the CA3-CA2-CA1. Additionally singly stained cell bodies are scattered across the hippocampus.





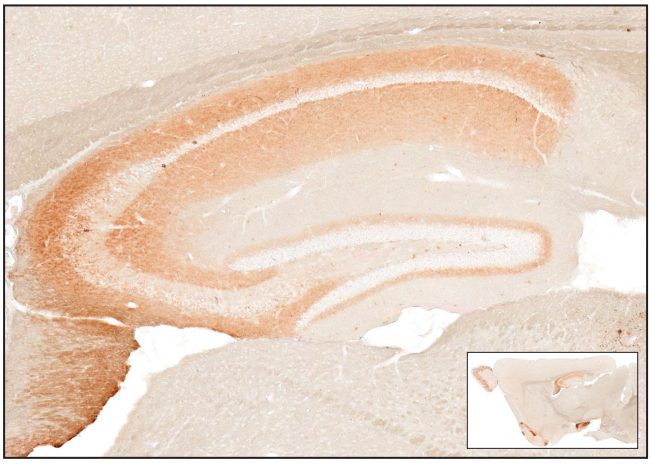


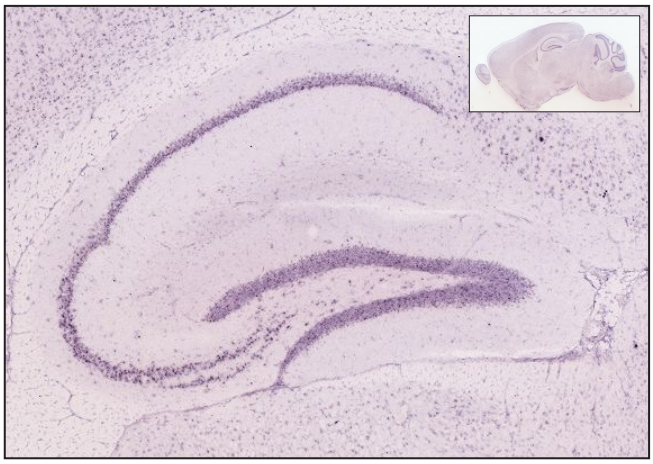


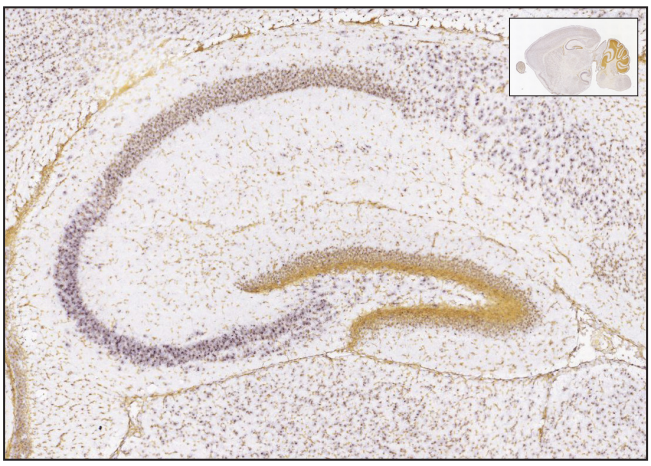


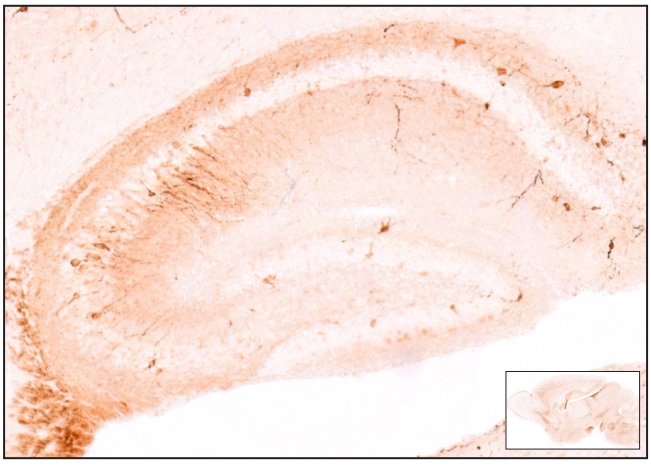
5HT5b	Image series Id
Allen adult	69257975
Allen P14	100071150

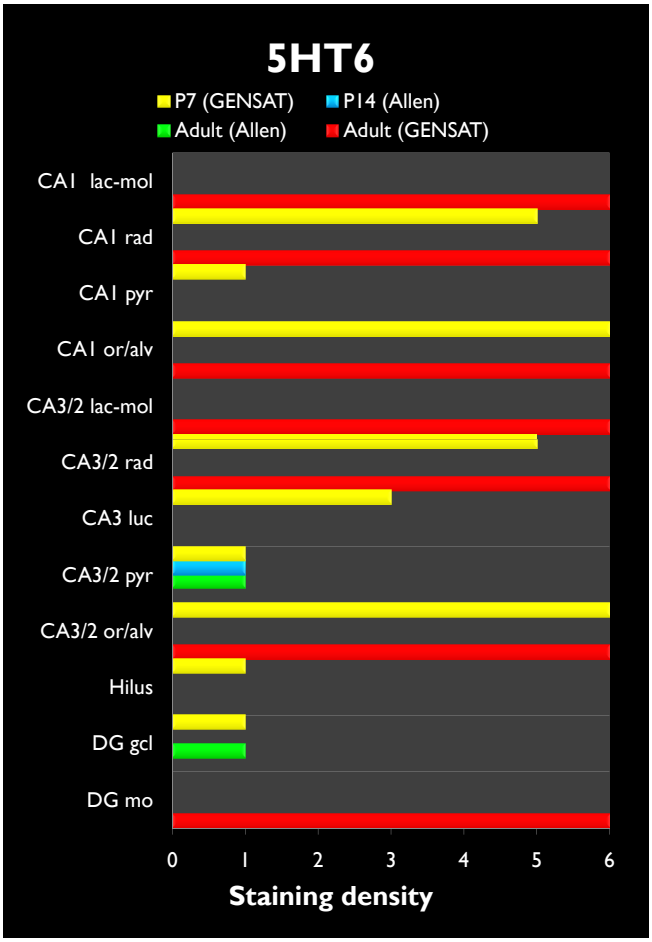
In the GENSAT adult there are few singly stained cell bodies in the dentate gyrus granule cell, and molecular layer, in the CA1 pyramidal layer and stratum oriens/alveus, as well as the CA3/2 stratum radiatum. Apart from that the series is unstained. Both the Allen adult and the Allen P14 show no staining. More interesting is the distribution in the P7 mice. This distribution appears complementary to the 5a. The inner molecular layer of the DG, the stratum oriens and stratum radiatum of CA3/CA2 and CA1 show ubiquitous staining. Note the lack of staining of the CA3 stratum lucidum and the hilus. Also note the overlap of the CA1 lacunosum moleculare in the 5a and 5b slides. The principal cell layers of all regions contain a few stained cells.





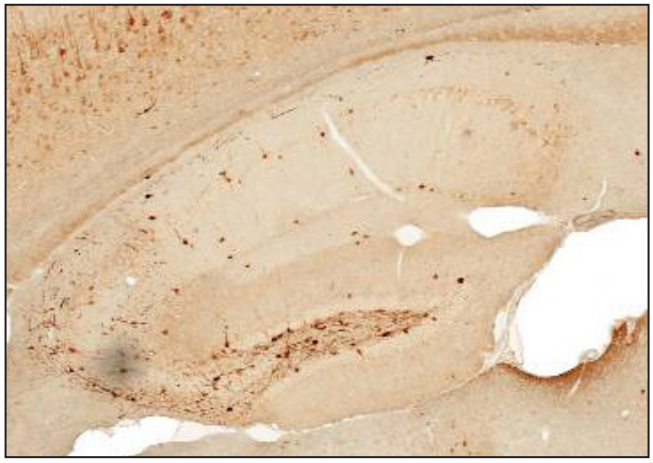


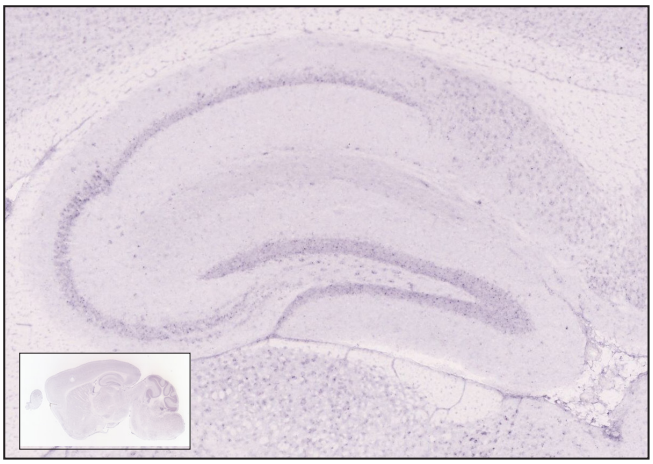


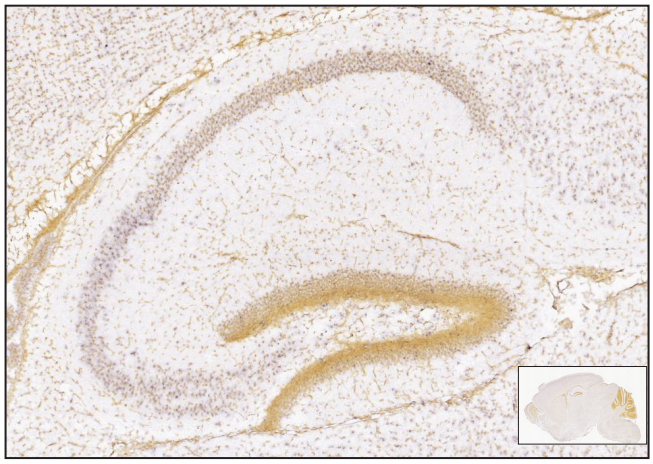


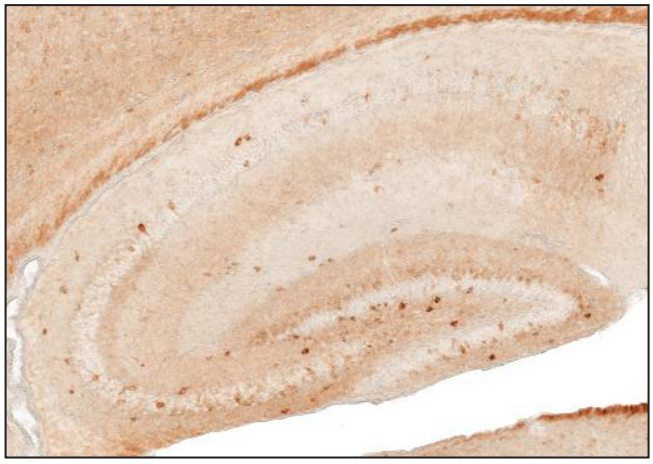
5HT6	Image series Id
Allen adult	69257981
Allen P14	100071115

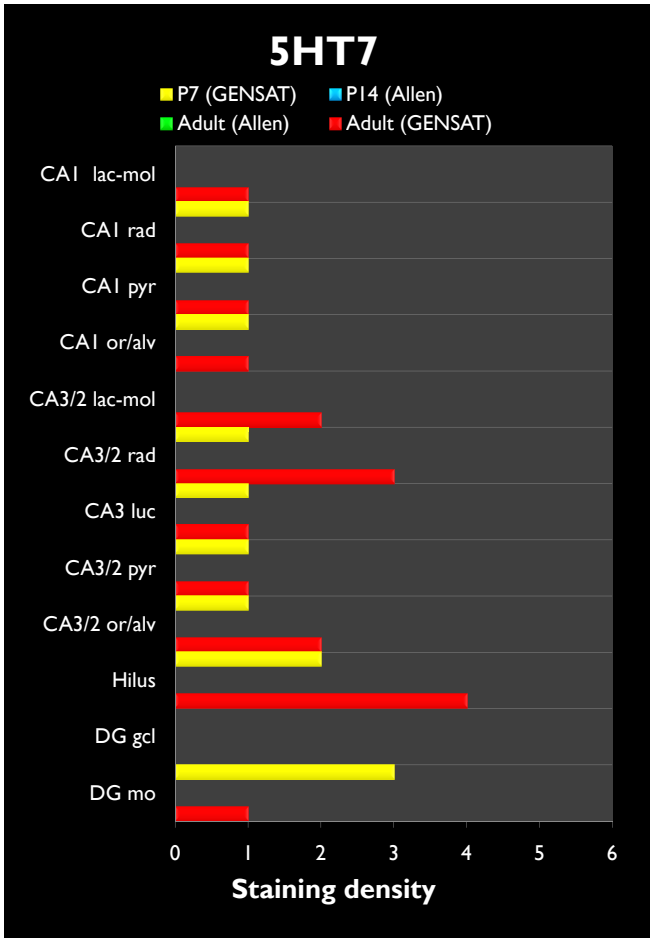
In the GENSAT adult series there is a ubiquitous staining of nearly all the molecular layers of the HC formation. Notable exceptions are the CA3 stratum lucidum and hilus. Also note layered staining of the inner molecular layer of the dentate gyrus. In the Allen adult series only singly stained cell bodies in the CA3/2 pyramidal layer and the dentate gyrus granule cell layer is evident. In the Allen P14, there are singly stained cell bodies in the CA3/2. The staining pattern of the GENSAT P7 series is similar to the adult GENSAT slides, with a few exceptions. Note the lower staining density of the CA1 stratum radiatum, CA2/3 stratum radiatum, CA3/2 stratum oriens/alveus and the lack of staining of the dentate gyrus molecular layer. Additionally there are scattered singly stained cell bodies in all principal cell layers of the HC, not present in the adult series.











5HT7	Image series Id
Allen adult	71393436
Allen P14	100071941

In the GENSAT adult series there are scattered staining in all layers except the dentate gyrus granule cell layer. Notable is the increased dense staining from the hilus through to CA3/2. Comparably the density is visibly reduced in the CA1. In both the Allen adult and P14 series there is no discernible staining of the hippocampus. In the GENSAT P7 series, there is a similar distribution pattern to the GENSAT adult series, but with a more anatomically constrained expression. Note however that the relatively dense staining of the hilus and CA3, and the scarcer staining of CA1 is also present here.

5.0 Discussion

A purpose of this study is to find which sub-receptors of the dopaminergic and serotonergic is found in the adult and developing hippocampus in the mouse brain. There appear not to be one subregion specific dopamine or serotonin sub-receptor. Most of the receptors investigated here are expressed in the murine hippocampus. Many of the receptors are also present in all the hippocampal subregions, but with a scarse, seemingly random distribution. Some of the receptors display a dense distribution pattern on the principal cells of the hippocampus. The other purpose is to use the anatomical distribution of the staining pattern to determine which sub-receptors might be expressed on the adult-born newborn neurons of the subgranular zone in the dentate gyrus. A few of the receptors show a layered staining of the subgranular zone and may be promising candidates.

5.1 Most receptors are expressed on interneurons

One pattern that emerges from the above results are that most of the subreceptors are expressed on scattered cells in the molecular layer. These probably correspond to hippocampal interneurons (van Strien et al., 2009). This may seem like an excessive expression pattern, but it could enable a very precise and context-specific modulation of hippocampal functions. In the case of memory, it could be achieved by lowering general hippocampal inhibition to increase the probability of recording all salient information in a rewarding context (Aimone et al., 2010). There could also be an increase in inhibition that would block all memory formation not accompanied by an activation neuromodulatory receptors on the hippocampal principal cells, which are also present. If the receptor distribution here is representative of dopaminergic and serotonergic regulation of murine hippocampal functions it seems certain that untangling the precise role of each subreceptor in the hippocampus will be daunting.

5.1 Expression in the murine CA1

The CA1 pyramidal layer is most notably stained by the D3 and D5 dopamine receptors, and the 5HT1a, 5HT1b, 5HT1f serotonergic receptors. D3 also has a dense staining of the CA1 molecular layers in the GENSAT adult series, indicating that the D3 is important in the murine CA1. The 5ht6 receptor show a dense staining of all CA1 molecular layers in the GENSAT adult series.

5.2 Expression in the murine CA3

The CA3 pyramidal layer are only densely stained by the 5HT1f receptor. Across all subreceptors the CA3 are usually the least stained. A notable exception is the stratum lucidum, as seen in the 5HT4 receptor, however these are most probably mossy fiber projections from the dentate gyrus granule cells. One interesting distribution can be seen by the staining of the 5HT7 receptor, which has a near continuos band of scattered stained cells from the hilus to the CA3 pyramidal layer.

5.3 Expression in the murine dentate gyrus

The 5HT1a, 5HT1f, and 5HT4 Show the densest staining of the dentate gyrus granule cell layer. When comparing the expressed receptors in the dentate gyrus, it appears to be the second most regulated site in the hippocampus. Interestingly the granule cell layer staining pattern sometimes have a layered pattern, e.g. only staining of the ventral blade, or a band in the basal granule cell layer, indicating a functionally distinct neuromodulatory regulation.

5.2 A possible regulator of adult neurogenesis

The D3 receptor shows a layered staining of the subgranular zone in the GENSAT adult series. This could implicate D3 in the regulation of adult neurogenesis in the mouse hippocampus. This receptor has been tied to adult neurogenesis in rats, but not in mice (Baker, et al., 2005). Since there have been demonstrated interspecies differences in receptor expression before, it is possible that this is exclusive to the GENSAT transgenic mouse, or that in these cells the D3 receptor do not have neuroregulatory functions (Platel et al., 2010). Another receptor implicated in adult neurogenesis is the 5HT4 receptor (Djavadian, 2004). In this study the expression pattern of the 5HT4 receptor indicate staining of large parts of the granule cells of the dentate gyrus, strengthened by the dense staining of mossy fiber projections to the CA3 stratum lucidum. It is possible that the

staining pattern seen here can be reconciled with 5HT4 regulation of adult neurogenesis. One possibility is that immature neurons express 5HT4 and continue to do so as they mature. Another possibility is that the 5HT4 receptor regulate adult neurogenesis indirectly through the modulation of mature granule cell.

5.1 Expression profiles differ in the two databases

As seen above, there is a clear difference in expression pattern between the two databases. Partly, this can be attributed to the different methods employed by the databases. In the ISH study, the markers are confined to the neuron cell body, while the EGFP protein coexpressed with the target sub-receptor can also be found in the cellular neurites. As the EGFP is not tied to the receptor protein, the distribution pattern seen in the results are not representative for the distribution of the receptors on the neuron cell body. However the distribution of EGFP in the molecular layer are indicative of which neuronal subpopulations that transcribe the genes for the target receptor. The staining of the molecular layers of the HC may have extrahippocampal sources, as neurons from the entorhinal cortex and subiculum project to the dentate gyrus, but also directly to the CA3 and CA1 (van Strien et al., 2009). That would mean that the apparent difference in expression pattern between the databases are not as fundamental.

Another possible explanation for the difference between the databases may be that the transgenic mouse line has a different subtype expression than that of the C57/BL6 mouse. The great variety of serotonin receptors present in the hippocampus could represent a very precise functional modulation. It could also be argued that the great variety seen in the subtype expression may indicate that instead of a highly precise modulation by serotonin through these receptors, there is great redundancy. That could imply great variety in the receptor subtype expression profile as seen in other studies, in turn the transgenic mice may have a different sub-receptor expression profile to that of the C57/BL6 mice (Baker, et al., 2005).

Last there is naturally the danger of measurement bias, or erroneous classification of staining. Most of the staining registered here is of scattered singly stained cell bodies. While there may indeed be the case that there is a strong expression on hippocampal interneurons, there is also the possibility that they are mistermed.

Utility of results in further research

The expression mapping of receptors by mRNA and BAC coexpression is an indirect measure of the receptor proteins present at the cell surface. With a better understanding of which subreceptors are expressed in which neuronal population, it is easier to understand the role the neuromodulators have on these cells. Additionally the results found in this study could be used to look at the interplay of neuromodulatory receptors inside discrete regions of the hippocampus.

6.0 Conclusion

This study have investigated the distribution of serotonin and dopamine subreceptors in the murine hippocampus in two publicly available mouse brain databases. The main findings are that the receptor expression in the dopamine and serotonin neuromodulatory systems show great variance in murine hippocampus. In both the GENSAT and Allen brain map database, the number of dopamine and serotonin receptors expressed in discrete anatomical regions is great. Only a single receptor does not show any apparent expression in the murine hippocampus. Most of the receptors are expressed on scattered cells in the molecular layers, a position indicative of inhibitory interneuons. In the principal cell layers the number of densely expressed receptors are far less.

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