Report

Hippocampal Cholinergic Neurons in Developing CHATeGFP Reporter Mice

Boston University
Department of Pathology and Laboratory Medicine
Boston, Massachusetts

Austrian Marshall Plan Foundation

Author: Jasmine Rinnofner
Supervisor: Dr. Krzysztof Blusztajn

For David,
and all the mountains you gave to me
Rest in Peace

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1. Introduction

1.1. Alzheimer's Disease

The umbrella term "Dementia" describes the clinical manifestations of a variety of diseases and conditions whose underlying mechanisms includes brain nerve cell death or malfunction. Alzheimer's Disease (AD) is the most common type of dementia, contributing for 60 – 80% of all dementia cases. AD is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory decline and progressive impairment of activities of daily living, as well as a variety of neuropsychiatric symptoms and behavioral dysfunctions. Several warning signals for AD are known, such as memory loss that disrupts daily life, challenges in planning or solving problems, confusion understanding time or place and changes in mood and personality (http://www.alz.org/downloads/facts_figures_2012.pdf).

This progressive neurodegenerative disorder has a mean duration of around 8.5 years between beginning of clinical symptoms and death. In the mid-1970s studies report substantial neocortical deficits in the enzyme responsible for the synthesis of acetylcholine (ACh), choline acetyltransferase (ChAT). These results and the loss of cholinergic perikarya from the nucleus basalis of Meynert indicated a presynaptic cholinergic deficit which led to the cholinergic hypothesis of Alzheimer's disease (Francis et al., 1999). Besides the cholinergic hypothesis several other theories concerning the etiology and the pathogenesis of AD have emerged in the last years. Beta-amyloid and tau hypotheses are regarded as the mainstream ones. Other hypotheses that address various aspects of AD pathophysiology include the role for apolipoprotein E, cobalaminergic defiency and inflammation.

A decline in multiple cognitive functions is frequently associated with aging. Especially the ability to form memories of recent events and assimilate new and complex information tends to decline. Furthermore, these cognitive defects are indications of destructive, age-associated dementias such as AD. The major player in etiology of AD is amyloid precursor protein (APP) and its proteolytic derivative Aβ. Senile plaques and neurofibrillary tangles in medial temporal lobe structures and cortical areas of the brain in addition with degeneration of the neurons and synapses are the neuropathological hallmarks of AD. Senile neuritic plaques and blood vessels with amyloid angiopathy consist of insoluble β-amyloid (Aβ), while abnormally hyperphosphorylated forms of the microbubule-associated protein tau are the main component of neurofibrillary tangles. Together with dystrophic neurites and reactive astrocytes those symptoms represent the clinical picture of AD (Bayer et al., 2001). Synaptic dysfunction in AD brain is caused by the accumulation of soluble Aβ oligomers (AβOs) at excitatory

synapses. Nunes-Tavares et al. (2012) showed that nanomolar concentrations of AβOs induce a considerable inactivation of neuronal ChAT. Furthermore, polyunsaturated fatty acids blocked ChAT inhibition by AβOs. Polyunsaturated fatty acids are considered to be efficient antioxidants which indicates that oxidative damage may be involved in ChAT inhibition.

Lesions in the septohippocampal cholinergic system are thought to contribute to characteristic features of Alzheimer's Disease, e.g. cognitive decline of old age and memory loss. Studies of patients who suffered from AD have found damage or abnormalities in these cholinergic pathways. This cholinergic hypothesis states that a loss of cholinergic function in the central nervous system contributes to the cognitive decline associated with AD (Terry and Buccafusco, 2003).
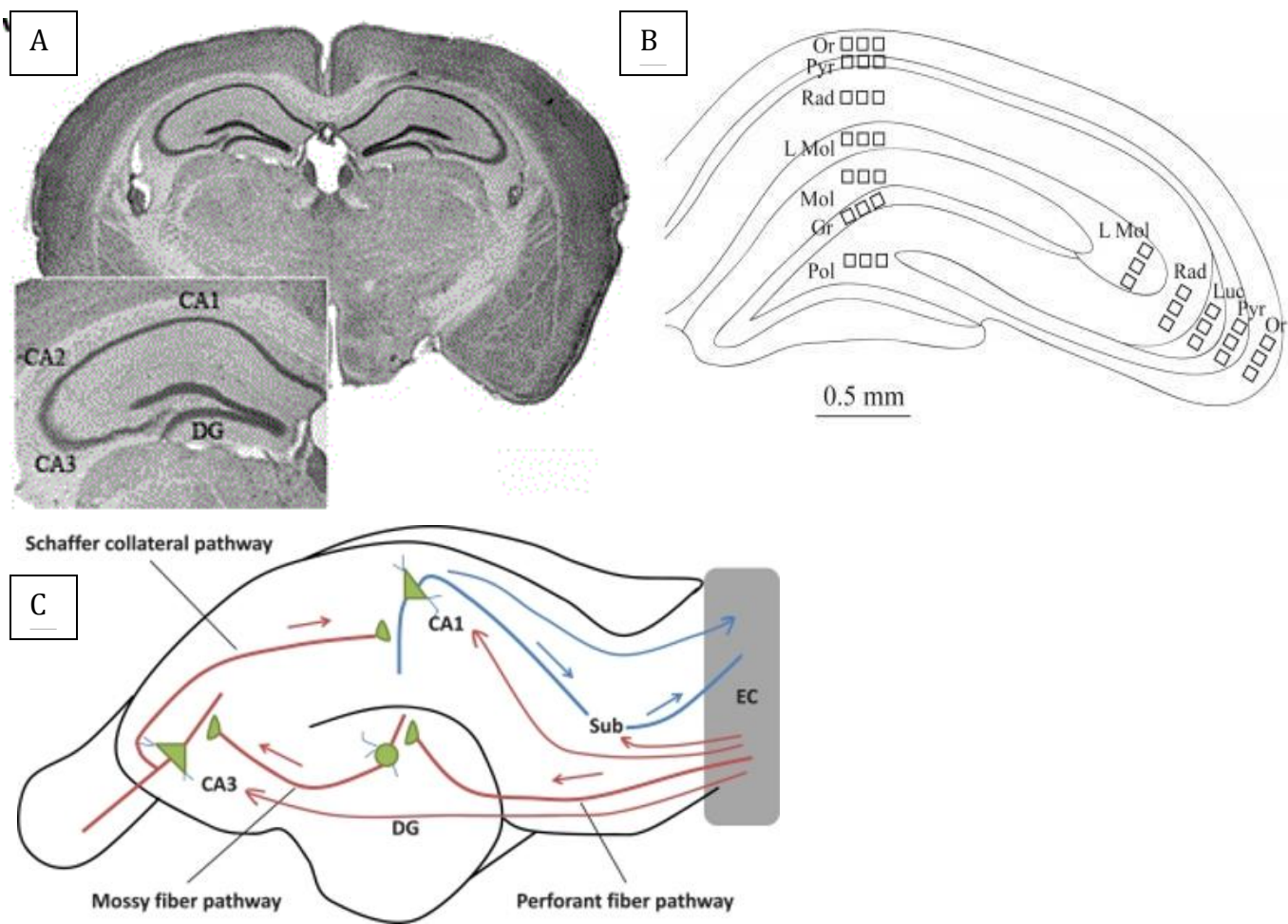
1.2. The hippocampus

The hippocampus lies deep within the medial lobe of the human brain and is one of the most widely studied regions of the brain. The hippocampus itself is part of several related brain regions that together are called the hippocampal formation. The hippocampus proper (cornu ammonis) can be divided into three subdivisions: CA1, CA2 and CA3. The other regions of the hippocampal formation are the dentate gyrus, subiculum, presubiculum, parasubiculum and entorhinal cortex (Andersen et al., Chapter 3, 2006). The hippocampus proper consists of several layers: Alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare and stratum lucidum. The dentate gyrus is composed of three layers: molecular layer, granular layer and polymorphic layer (hilus). (http://neuralnetoff.umn.edu/mediawiki/index.php/Hippocampal_Anatomy)

The entorhinal cortex provides the major input to the hippocampus and in addition other parts of the brain (e.g. amygdala, orbital cortex, olfactory bulb, cingulate cortex) have inputs to the hippocampus via the entorhinal cortex. A second source of input to the hippocampus is the precommissural and postcommissural branch of the fornix. While the precommissural branch of the fornix originates from the nucleus basalis of Meynert, the postcommissural branch comes from the hypothalamus. Information, that passes through the hippocampus get processed by the hippocampus proper and the dentate gyrus. From the dentate gyrus connections are made to CA3 of the hippocampus through mossy fibers and to CA1 via Schaffer collaterals. From these two CA fields information goes through the subiculum and enters the alveus, fimbria and fornix and then passes to other areas of the brain. A coronal overview of

the hippocampal formation including the specific layers and pathways within the hippocampus is given in figure 1 (http://neuroscience.uth.tmc.edu/s4/chapter05.html).

The hippocampus belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory, spatial navigation and control of attention. In animal models hippocampal function is studied using a range of learning tasks, e.g. trace conditioning, contextual fear conditioning, social transmission of food preference, object recognition and spatial navigation. In AD, the hippocampus is one of the first regions of the brain to suffer damage (Leuner and Gould, 2010).

http://www.thequantumlobechronicles.blogspot.com
http://www.nicolasaznavour.com/image-gallery/#nogo
http://openi.nlm.nih.gov/imgs/rescaled512/3131729_CN-9-388_F1.png

Figure 1: coronal overview of the hippocampal formation. A: the picture shows the regions CA1, CA2, CA3 and the dentate gyrus (DG) of the anterior hippocampus. B: the picture shows the different layers within the hippocampus: Oriens (Or), pyramidal layer (Pyr), stratum radiatum (Rad), stratum lacunosum moleculare (LMol), molecular layer of DG (Mol), granular layer of DG (Gr), polymorph or hilus of DG (Pol), lucidum (Luc). C: overview of the fiber pathways in the hippocampus is shown; sub (subiculum), EC (entorhinal cortex).

1.3. The central nervous system: neurotransmission and cholinergic neurons

The central nervous system consists of two types of specialized cells: neurons and glia. While neurons are the structures processing basic information, glia cells provide a structural framework that allows networks of neurons to remain connected. Generally speaking, the neuronal network is composed of three kinds of neurons: motor neurons, sensory neurons and interneurons. Each type of neuron possesses distinct parts: a soma and processes including axon and dendrites. While the neuron receives its incoming signal from other neurons through its dendrites, the outgoing signal flows along its axon. To achieve rapid long distance communication between cells electrical signals (action potentials) are sent along axons. The axon terminals are the structures that contain neurotransmitters - chemical substances through which signals flow from one neuron to the next via chemical synapses. The movement of chemicals or electrical signals across a synapse to provide communication between neurons is accomplished by neurotransmission. Electrical neurotransmission and chemical neurotransmission are two different ways how information can be send from one neuron to another. A narrow gap between pre- and postsynaptic neurons allows neurotransmission at an electrical synapse while chemical neurotransmission occurs at chemical synapses and requires a neurotransmitter. The pre- and postsynaptic neurons are separated by a small gap, the synaptic cleft and neurotransmitter link the action potential of one neuron with a synaptic potential in another to forward the electric signal. (http://www.mind.ilstu.edu/curriculum/neurons_intro/neurons_intro.php)

Many neurotransmitters such as Acetylcholine, Norepinephrine, Dopamine, GABA, Glutamate, Serotonin and Endorphin are known. Acetylcholine was the first neurotransmitter discovered by Otto Loewi in 1921 (http://webspace.ship.edu/cgboer/genpsyneurotransmitters.html). Both, in the peripheral nervous system and central nervous system acetylcholine (ACh) acts as a neurotransmitter. ACh is part of the cholinergic system and cholinergic neurons in the central nervous system are one type of neurons which use this neurotransmitter for neurotransmission. ACh is synthesized by the biosynthetic enzyme ChAT. The single step reaction in ACh synthesis involves the availability of acetyl-CoA and choline. Choline transporters (CHT) are necessary to supply the neurons with choline by catalyzing the

uptake of this nutrient from the extracellular space to the neuronal cytoplasma. Only a small amount of ACh is free in the cytosol, the majority is contained in vesicles. Therefore the vesicular ACh transporter (VACHT) packages the ACh into secretory synaptic vesicles before ACh gets released into the synaptic cleft by Ca^{2+} stimulated docking and fusion of the vesicle with the neuronal cell membrane. After stimulation of acetylcholine receptors (AChR) on the post-synaptic membrane, acetylcholine esterase (AChE) breaks down ACh into choline and acetate (figure 2). (http://neuroscience.uth.tmc.edu/s1/chapter11.html).
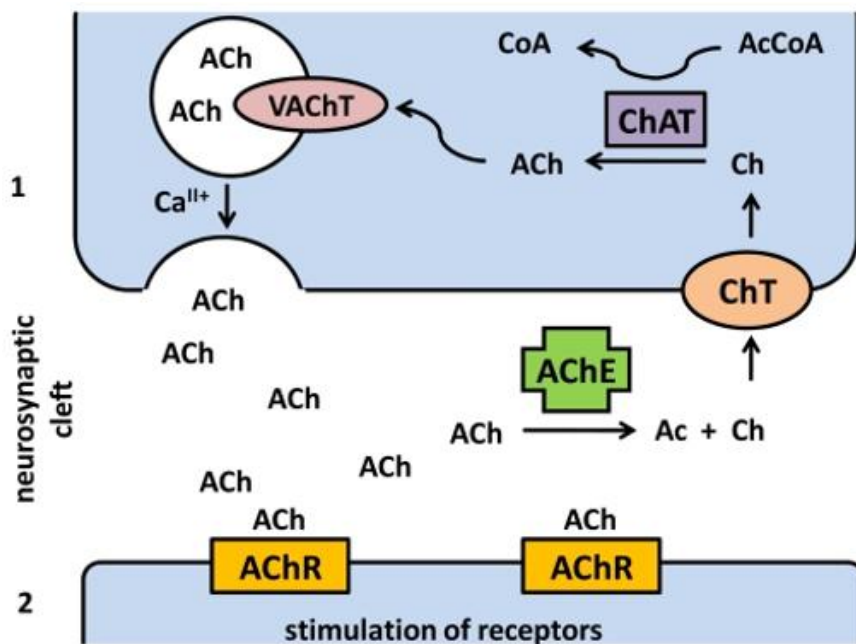
**Figure 2**: *Synthesis, storage and release of ACh in cholinergic neurons.*
http://openi.nlm.nih.gov/imgs/rescaled512/3292018_ijms-13-02219f1.png

Based on the chemical agents nicotine and muscarine, two types of receptors for ACh are known on the postsynaptic membrane: nicotinic and muscarinic receptors. The first one is linked to ionic channels while the latter is linked to a 2nd messenger system through G proteins (http://neuroscience.uth.tmc.edu/s1/chapter11.html).

Cholinergic neurons have significant importance in the process of learning, memory, attention and sleep. The basal forebrain cholinergic complex which includes medial septum, horizontal and vertical diagnonal band of Broca and nucleus basalis of Meynert send cholinergic projections to the cortex and hippocampus. The cholinergic neurons in this complex undergo moderate degenerative changes during

aging. Either cell loss or dendritic, synaptic and axonal degeneration might contribute to this degenerative process. The result of these changes leads to a cholinergic hypofunction that has been related to the progressing memory deficits with aging (Schliebs and Arendt, 2011). The innervation of the hippocampus and cerebral cortex by basal forebrain cholinergic neurons (BFCN) is one of the key components of the neuronal circuitry necessary for learning and memory. BFCNs provide modulatory input mediated by the neurotransmitter ACh. Both, in aged humans and animals and in AD patients and AD animal models a decline in BFCN function and reduced cholinergic marker expression is evident (Mufson et al., 2008).

Many studies have confirmed that the main cholinergic input to the hippocampus emanates from neurons in the medial septal/diagonal band complex and the hippocampal formation has been widely used for the study of the cholinergic system (Frotscher et al., 1986; Matthews et al., 1987). Studies using retrograde tracing with acetylcholinesterase histochemistry or choline acetylransferase immunocytochemistry have shown that about 50% of all projections from the medial septum/diagonal band to the hippocampal formation are cholinergic (Senut et al., 1989; Baisden et al., 1984; Woolf et al., 1986). Evidence to support the cholinergic nature of these septohippocampal projections has arisen from lesion studies followed by neurochemical measurements of the cholinergic markers AChE and ChAT. Septal lesions result in decreases of these markers in the hippocampus and fascia dentata (Lewis e. al., 1967). Non-cholinergic as well as cholinergic neurons in the medial septum and vertical limb of the diagonal band send their axons to the hippocampus. The regions CA2, CA3 and the hilar region of the dentate gyrus (DG) receive their cholinergic input from the medial septum-vertical limb of the diagonal band complex. The cholinergic fibers deriving from this part pass through the fimbria and dorsal fornix to terminate in the stratum oriens and radiatum adjacent to the pyramidal cells in fields CA2 and CA3 and in the molecular layer of the DG. The pyramidal cells of the hippocampus and the granula cells of the DG show the highest AChE and ChAT activity in terminal fields (Fibiger, 1982). Two groups of cholinergic neurons are known in the basal forebrain: the medial septal group (medial septal nucleus and vertical diagonal band; ms and vdb) that project cholinergic axons to the hippocampus and parahippocampal gyrus. The other group of cholinergic neurons is the nucleus basalis group, consisting of nucleus basalis, substantia innominata and horizontal limb of the diagonal band (bas, si, hdb) that project cholinergic axons to all parts of the neocortex, parts of the limbic cortex and to the amygdala (see figure 3) (http://nwoolf.bol.ucla.edu/).
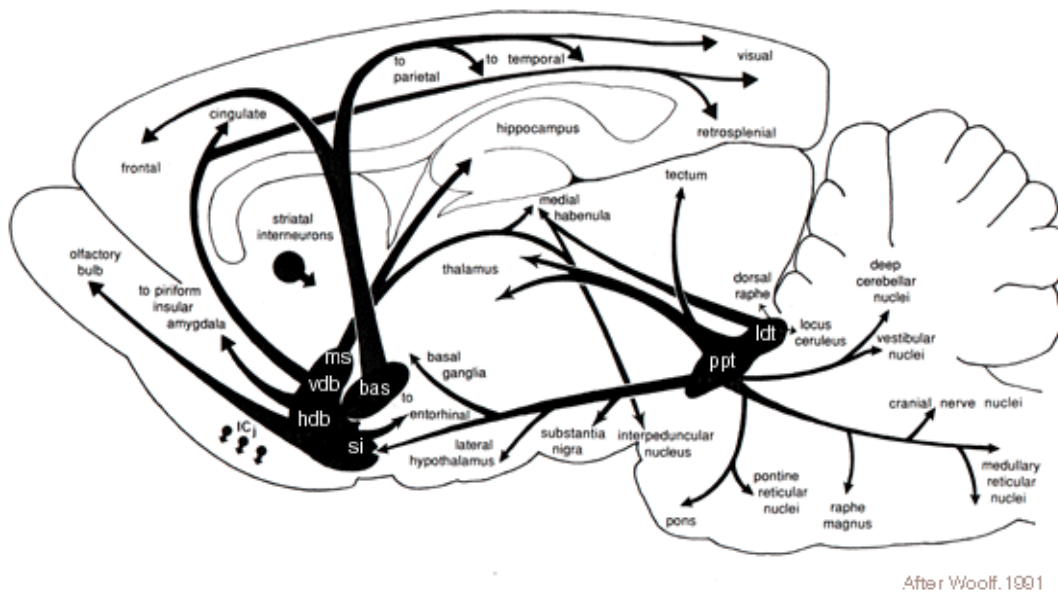
Figure 3: *Schematically image of the central projections of cholinergic cells on a sagittal section from rat brain. The entire cortical mantle is innervated by the basal forebrain cholinergic system.*

Immunohistochemical localization of ChAT has been used extensively to identify cholinergic neurons. However, the precise distribution and morphology of these neurons in the hippocampus have been controversial (Frotscher et al., 1986; Wainer et al., 1985; Matthews et al., 1987; Clarke, 1985; Kanaya-Ida and Ben Ari, 1989). Some investigators found ChAT-positive cells mainly in the stratum-lacunosum moleculare, on the border of stratum lacunosum moleculare and stratum radiatum and within the molecular layer of the dentate gyrus (Matthews et al., 1987; Frotscher et al., 1986), others report ChAT-positive neurons in the hilus of the dentate gyrus (Wainer et al., 1985; Kanaya-Ida and Ben Ari, 1989 and Blaker et al., 1988). This variability in distribution could be due to using different immunohistochemical techniques including various antibodies against ChAT. However, not much is known about the development, distribution, amount and morphology of cholinergic neurons in the hippocampal formation.

The aim of this project is to investigate the development of cholinergic neurons in brains of postnatal CHATeGFP mice. These mice are a transgenic strain generated with the use of a bacterial artificial chromosome containing the *Chat* locus. In this locus, the enhanced green fluorescence protein (EGFP) is under control of the *Chat* promoter which enables a detection of cholinergic neurons with the fluorescence microscope. CHATeGFP+/+ mice were used to detect the normal development of cholinergic neurons during the postnatal period. Mice of various ages were euthanized and their brains

were fixed, cryoprotected and cut with the microtome in two different ways: sagittal and coronal. Confocal images were taken from the hippocampus of each section. Moreover, cell count measurements were performed to obtain the total number of green fluorescent cells. Cells, which express EGFP were regarded as cholinergic neurons in this report. To confirm the presence of cholinergic neurons within the hippocampal formation, which remains controversial, immunohistochemistry of the three cholinergic markers ChAT, CHT and VACHT was performed.

1.4.Immunohistochemistry

Immunohistochemistry (IHC) is a histological technique based on antibody-antigen reactions. It combines anatomical, immunological and biochemical techniques to identify discrete tissue components. This technique allows visualization of the distribution and localization of cellular components within tissue sections or cells. IHC is used in diagnostic, drug development and biological research and samples can be viewed by both light and fluorescence microscopy. Complete preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. Therefore the fixation of tissues is an important step that crosslinks proteins and preserves cellular components. The most common fixative is formaldehyde. For detection of the antigen in the selected tissues two principles are known: a direct method and an indirect method. The direct method is a one-step staining method and involves a labeled primary antibody. Different labels are known, including fluorochromes, enzymes and biotin. Because this technique utilizes only one antibody it is simple and rapid but the sensitivity is lower due to little signal amplification. The indirect method is more sensitive compared to the direct method, because signal amplification leads to a better result. The indirect method uses an unlabeled primary antibody that binds to the target antigen in the tissue and a labeled secondary antibody that reacts with the primary antibody. The target antigens are detected by either chromogenic or fluorescence labeled antibodies. Chromogenic detection is based on enzyme activities such as horseradish peroxidase or alkaline phosphatase that form colored precipitates following the addition of substrate, most often DAB or NBT/BCIP (Ramos-Vara, 2005).

1.5.Confocal microscopy

To visualize the cholinergic neurons within the hippocampal formation a laser scanning confocal microscope from *Zeiss* was used. Usually, in a fluorescence microscope all parts of the sample in the optical path are excited at the same time because the entire specimen is flooded in light from a light source. This leads to a large unfocused background during the detection of the resulting fluorescence. Compared to conventional fluorescence microscopes a confocal microscope uses point illumination and a pinhole as filter techniques to eliminate out-of-focus signals. Only light produced by fluorescence that is very close to the focal plane can be detected and this results in a better optical resolution and contrast of the image. Light from a laser got reflected by a dichromatic mirror and get passed on the specimen through the objective. The microscope excludes out-of focus flare that occurs in thick fluorescently labeled specimens. Light points from the specimen are detected by a photomultiplier tube (PMT) through a pinhole and the output of the PMT is built into an image and displayed by the computer (figure 4). Another advantage of confocal imaging is the possibility to take high-resolution images in sequences through thick sections or even whole-mount specimens. Digital image processing methods can be applied and allow z-series and three-dimensional illustration of objects. Although the lasers are high-intensity monochromatic light sources, two or more fluorescence emission signals can overlap in digital images due to their close proximity within the specimen. Furthermore the emission of one fluorophore being detected in the photomultiplier channel reserved for a second fluorophore causes bleed-through artifacts. This fundamental problem known as crossover must be considered when taking confocal images.
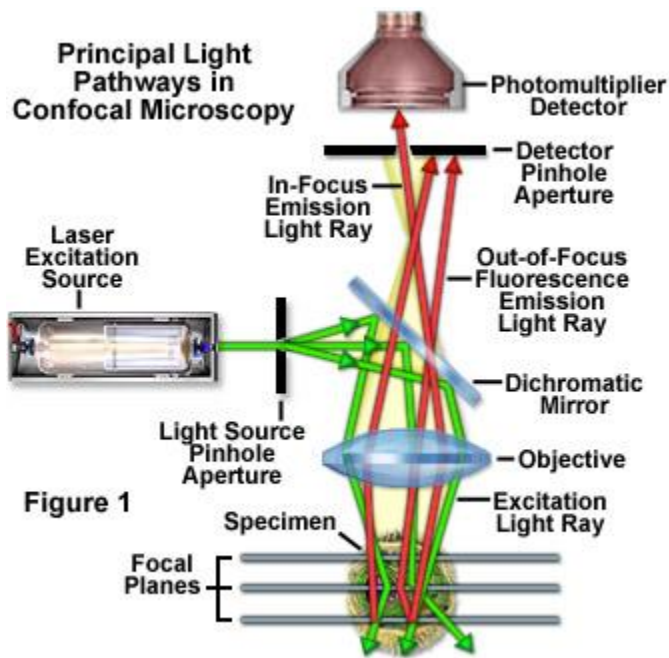
(http://micro.magnet.fsu.edu/primer/techniques/confocal/index.html)

Figure 4: *Principal light pathways in confocal microscopy. Only emission light rays that are in the focal plane reach the photmultiplier through a pinhole and are built into an image. This techniques enables the production of a high resolution image.*

Techniques used in this project include z-series through multiple planes in one specimen and tile scanning (mosaic scans). Taking mosaic scans of large images gives you the advantage of getting a high resolution picture from a wide area within the tissue sample. Several images are taken to cover the whole area of interest and are put together to form a big picture.

2. Materials and Methods

CHATeGFP+/+ mice (B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J) were used for investigation of cholinergic neurons in developing brains. Mice were euthanized with $CO2$ and decapitated at the ages of P4, P7 (first postnatal week), P14, P21 (second and third postnatal week), P32 and P40 (fifth and sixth postnatal week) and 6 months (1 mouse per age). Brains were carefully removed with scissors and forceps and fixed in periodate-lysine/2%paraformaldehyde (PLP) at 4°C overnight. The next day brains were cryoprotected in 10% glycerol/2% DMSO solution in 0.1M phosphate buffered saline (PBS, pH7.4) for 24 hours and subsequently the brains were placed in a 20% glycerol/2% DMSO in 0.1M PBS solution.

Brains were sectioned 40μm (80μm at P4) either coronal or sagittal with a microtome from the *American Optical Company*, Instrument Division, Buffalo 15, New York, Model 860, serial number 24499. The knife was obtained from *C.L. Sturkey Inc,* Lebenon Pennsylvania. Sections were stored in 0.05% sodium azide/PBS at 4°C.

Sections from anterior hippocampus were selected for imaging.

Brain sections were mounted on subbed slides. Subbing solution contains 500ml ddH2O, 2.5g gelatin, 0.25 chromium potassium sulfate. The solution was heated to 60°C, filtered and microscope slides were subbed 30 minutes followed by a drying step in the incubator. Sections were mounted with *VectaShield* mounting solution, coverslipped and allowed to harden overnight at 4°C. Slides were sealed with nail polish and imaged with the confocal microscope. For cell count measurements 4 slices of the same brain were used to count the total number of cholinergic cells in the hippocampus. Weakly visible and too small cells were excluded from counting and the average and standard deviation were calculated in order to draw a graph. The distribution of cholinergic neurons in the hippocampus was investigated at 4 sliced of the same brain from the ages P4, P21 and P40. Imaging was performed on an LSM710 NLO laser scanning confocal microscope (Zeiss) using Argon/Krypton and Helium/Neon lasers set to 488nm and 594nm respectively.

For CHT, VACHT and ChAT immunofluorescence following antibodies were used: polyclonal mouse-anti-CHT (Invitrogen, 1:500), polyclonal rabbit-anti-VACHT (Invitrogen, 1:1000), polyclonal goat-anti-ChAT (Millipore, 1:20). Primary antibodies were diluted in PBS containing 0.3% Triton X-100, 0.2%

BSA, 0.1% 8% Na-azide and 5% normal donkey serum, normal goat serum or normal rabbit serum respectively. Free floating sections were removed from 0.05% sodium azide/PBS storage solution and rinsed with PBS. First the sections were incubated in blocking solution containing 10% donkey serum, goat serum or rabbit serum respectively and 0.3% Triton X-100 in PBS for 2 hours at room temperature. Afterwards sections stained for CHT and VACHT were incubated overnight and ChAT stained sections were incubated 48hrs at 4°C with primary antibodies. The next day, sections were rinsed with PBS (3 times for 10 minutes) and again blocked in aforementioned blocking solution for 2 hours. Then, sections were incubated at room temperature for 6 hours in the antibody buffer solution containing 5% donkey serum, goat serum or rabbit serum respectively in a solution supplemented with the corresponding secondary antibody. Following secondary antibodies were used: Alexa Fluor-594 rabbit-anti-goat IgG (Life Technologies, *Invitrogen*, 1:1000), Alexa Fluor-594 goat-anti-rabbit IgG (Life Technologies, *Invitrogen*, 1:1000) and Alexa Fluor-594 goat-anti-guinnepeg IgG (Life Technologies, *Invitrogen*, 1:1000). After the incubation sections were rinsed in PBS and allowed to dry on Superfrost*Plus* slides (Fisher), then coverslipped with VectaShield and stored at 4°C in the dark. An overview of the used antibodies and dilution is given in table 1.

	1° Antibody	Dilution	2° Antibody	Dilution
CHAT	Goat anti CHAT	1:200 and 1:20	Rabbit anti goat	1:1000
CHT	Mouse anti CHT	1:500	Donkey anti mouse	1:500
VACHT	Rabbit anti VACHT	1:1000	Goat anti rabbit	1:500

Table1: Antibodies for immunohistochemistry

Imaging was performed on an LSM710 NLO laser scanning confocal microscope (Zeiss) using Argon/Krypton and Helium/Neon lasers set to 488nm and 594nm respectively.

3. Results

Hippocampus: coronal sections

During the postnatal development an increase of about 0.5mm of the hippocampal size is detectable from P4 to P40. The layers of the hippocampus are hard to distinguish at the early ages, especially the stratum radiatum and stratum lacunosum moleculare are not yet separated at P4 and P7. Only the dentate gyrus is recognizable at those young ages. Additionally, the pyramidal layer seems to be thicker in P4 and P7 (about 8 to 9 cell layers) when compared to the older sections (about 3 cell layers). At P14 all hippocampal layers are clearly separated and distinguishable from each other and also the dentate gyrus is formed at around this age. Moreover, the shape of the hippocampal formation changes with age. While the hippocampus is more round and ovoid at P4 and P7, it gets its distinctive shape when the animals are 14 days (figure 5a).

The number of cholinergic neurons decreases significantly during development. Many cells expressing EGFP are found at P4 and P7 whereas only a few cells were found at P40 and in 6 month old mice (see graph 1). Moreover, the morphology of the cholinergic neurons changes. At young ages neurons appear rounder or sometimes polymorph in shape. Cholinergic neurons seem to be more ovoid and uniform at P40. Also the non-pyramidal cholinergic neurons appear to intercalate between the pyramidal neurons in the pyramidal layer at P4 and P7 (figure 8, 6a and 6b). At P4 cholinergic neurons are found throughout the whole hippocampus and dentate gyrus. They are mostly located in the stratum radiatum (on the border to the pyramidal layer), in the pyramidal layer itself, in the granular layer of the DG and in the hilus. No cells were found in the molecular layer of the DG. Because stratum radiatum and stratum lacunosum moleculare are not yet separated well it is hard to interpret in which layer the neurons are located. At P21 most of the cholinergic neurons are located in the pyramidal layer, stratum radiatum, border of stratum radiatum and stratum lacunosum moleculare and in the infragranular layer of the DG. Occasionally, cholinergic neurons were found in the stratum lacunosum and no cells could be observed in the molecular layer of the DG. The distribution of cholinergic neurons at P40 is similar to P21 whereas the number of cells is lower in general and there are fewer neurons in the pyramidal layer. In general, more cells seem to be in region superior (upper half of the hippocampus) (figure 5a and 5b).

Between P4 and P40, cholinergic fibers become thicker, longer, more organized, appear more continuous and connected to each other. The fiber network gets denser during this time. However, the

fiber density and thickness seem to decrease from P40 to 6 months old brains. Also at 6 month cholinergic fibers seem to be varicose. At young ages more cells than fibers could be obtained, while in older animals the fibers prevailed (figure 7a and 7b).

Hippocampus: sagittal sections

Cholinergic fibers deriving from the medial septum projecting via the fimbria-fornix to the hippocampal formation could be observed. Moreover, at P7 more cholinergic cells within the projecting fibers are seen. Additionally, it seems that there is a lack of eGFP positive fibers in the anterior commissure in all sagittal sections (figure 9).

Immunohistochemistry

Immunohistochemistry for VACHT stained cholinergic neurons in the medial septum, but no fibers in the hippocampus could be detected with the 594nm laser. Staining for CHT did not work at all (data not shown). ChAT positive cholinergic neurons could be obtained in the medial septum, cortex and striatum. In the hippocampus ChAT stained fibers colocalized with eGFP positive fibers (figure 10a). No ChAT positive cell bodies were detectable in the hippocampus. A slight shadow of ChAT positive (red) cells was visible in the same area where eGFP neurons were situated (figure 10b).
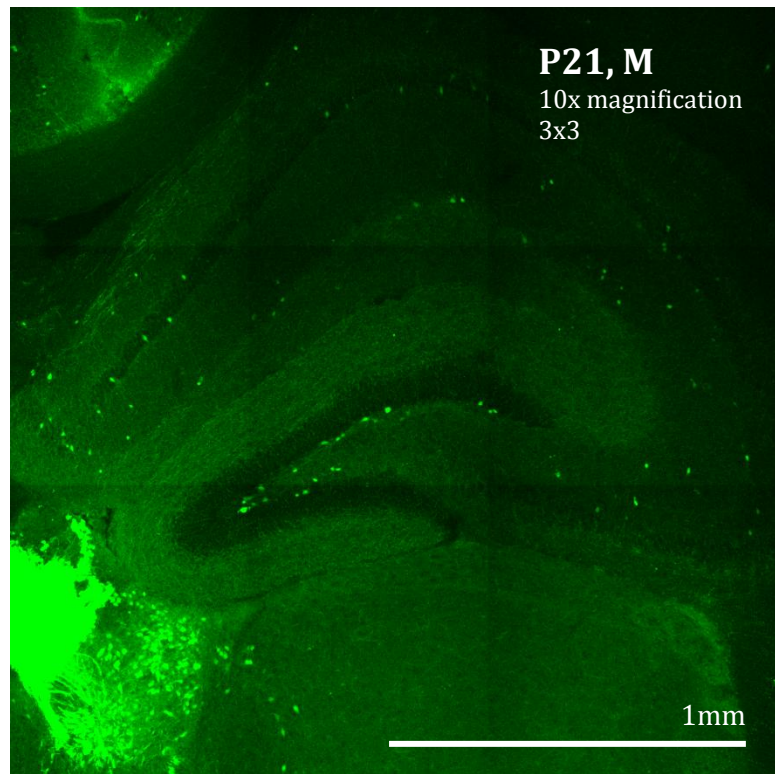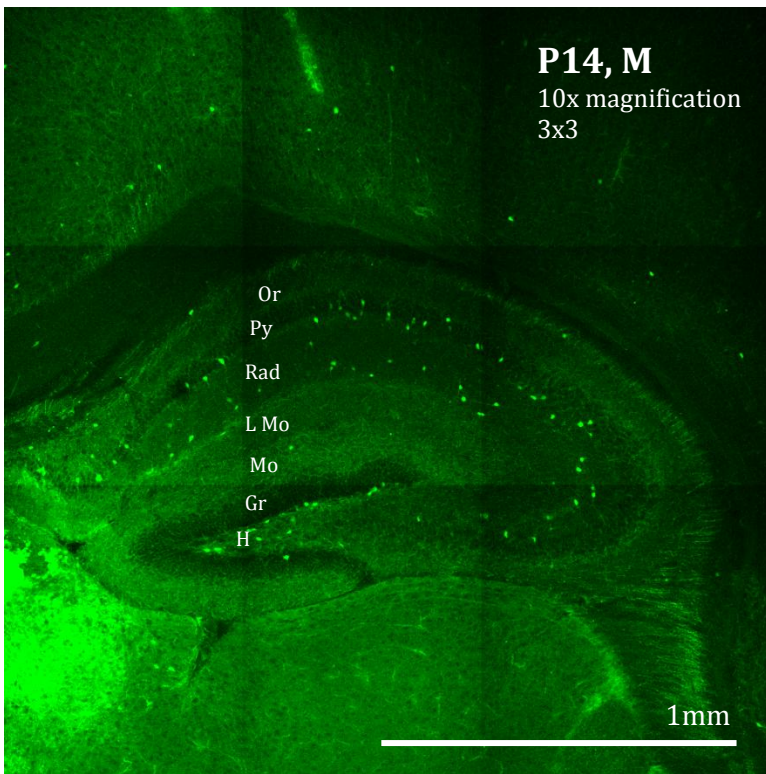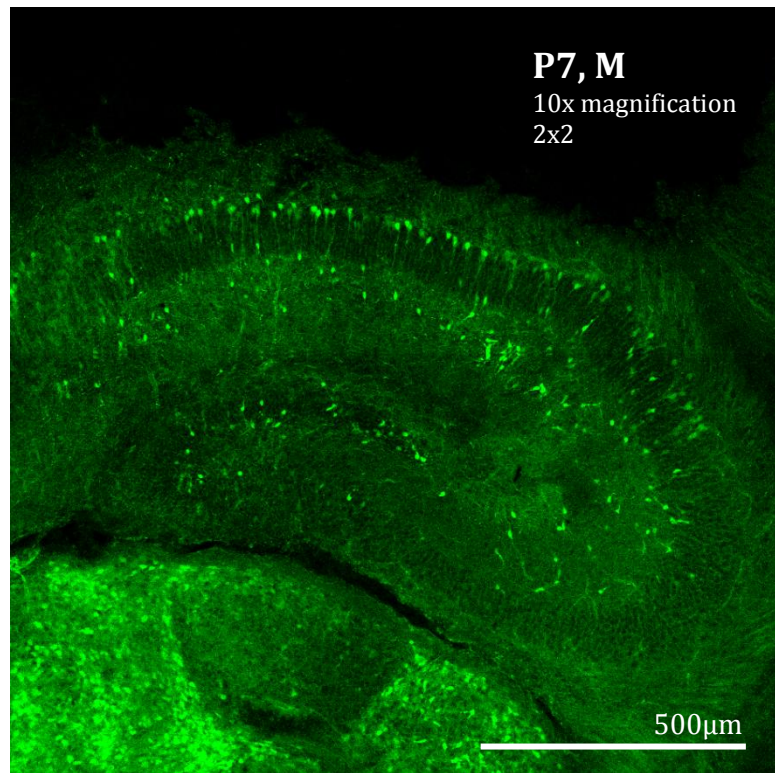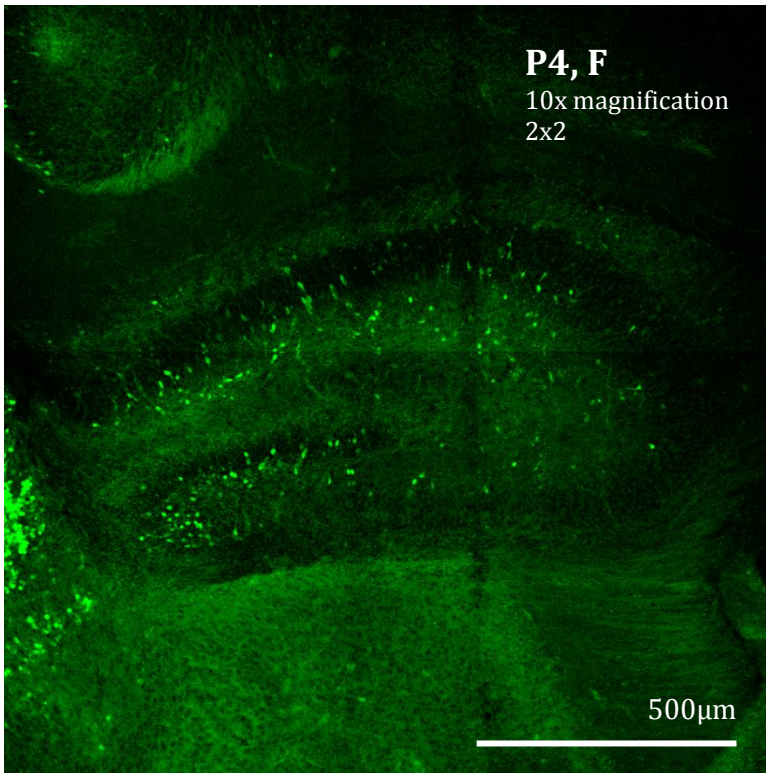
Figure 5a: coronal overview of the anterior hippocampus. Ages P4 to P21 are shown in a 10 fold magnification. Note how the DG is developing and how the layers of the hippocampus become distinguishable from P7 to P14. Cholinergic neurons are intercalating between pyramidal cells in the pyramidal layer in P7.

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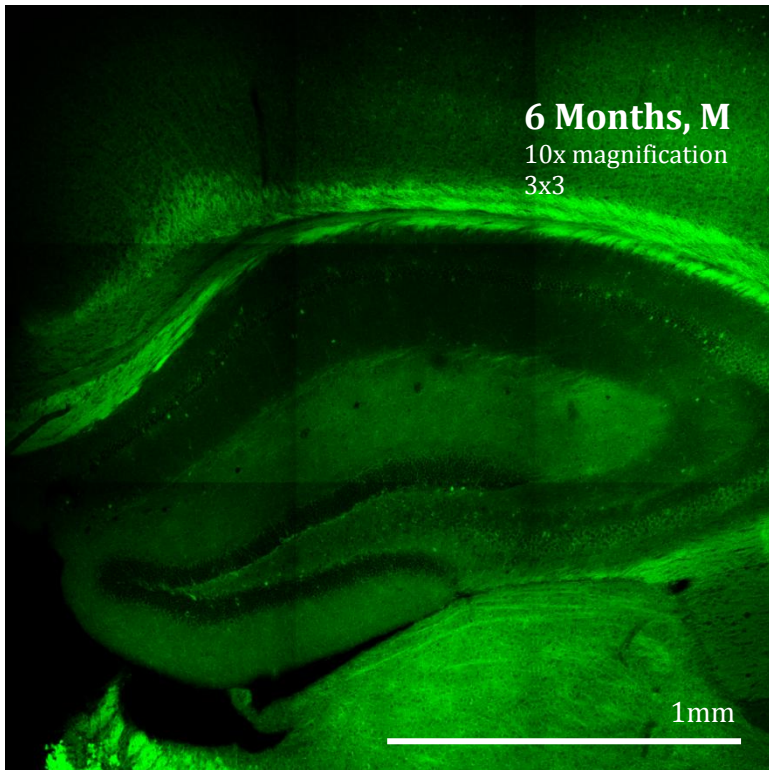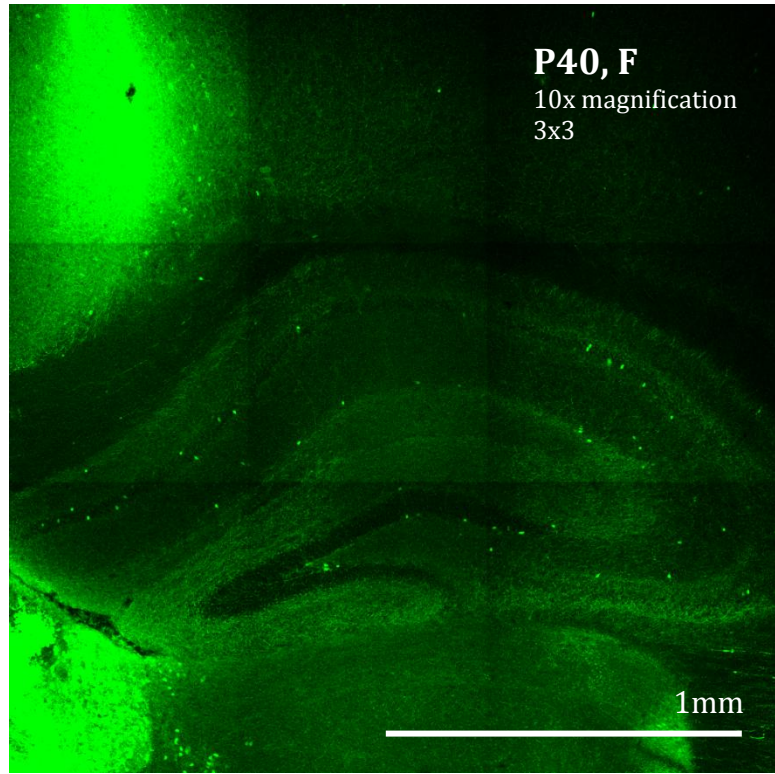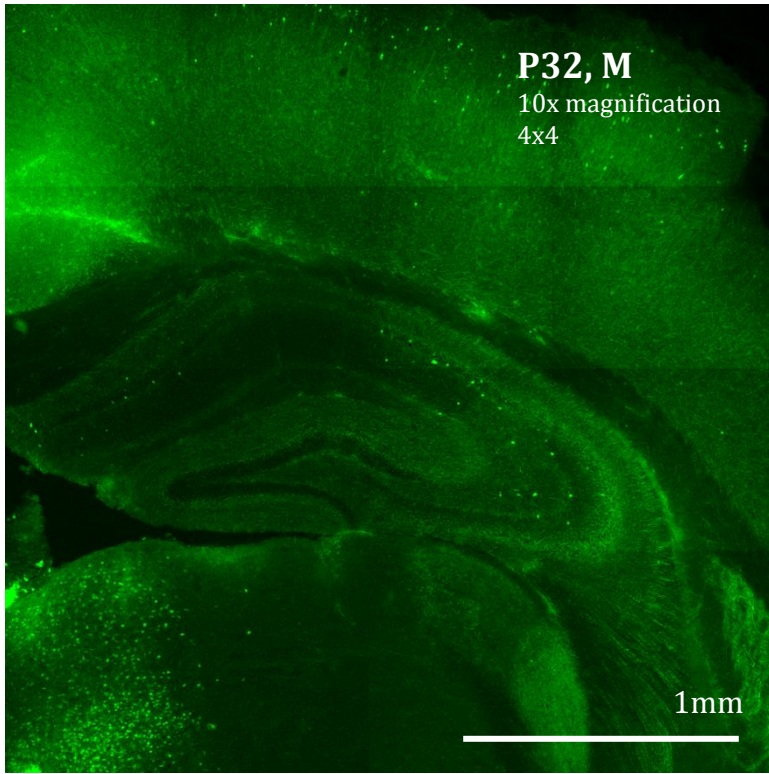
Figure 5b: *coronal overview of the anterior hippocampus. Ages P32 to 6 Months are shown in a 10 fold magnification. The amount of cholinergic neurons decreases with age.*
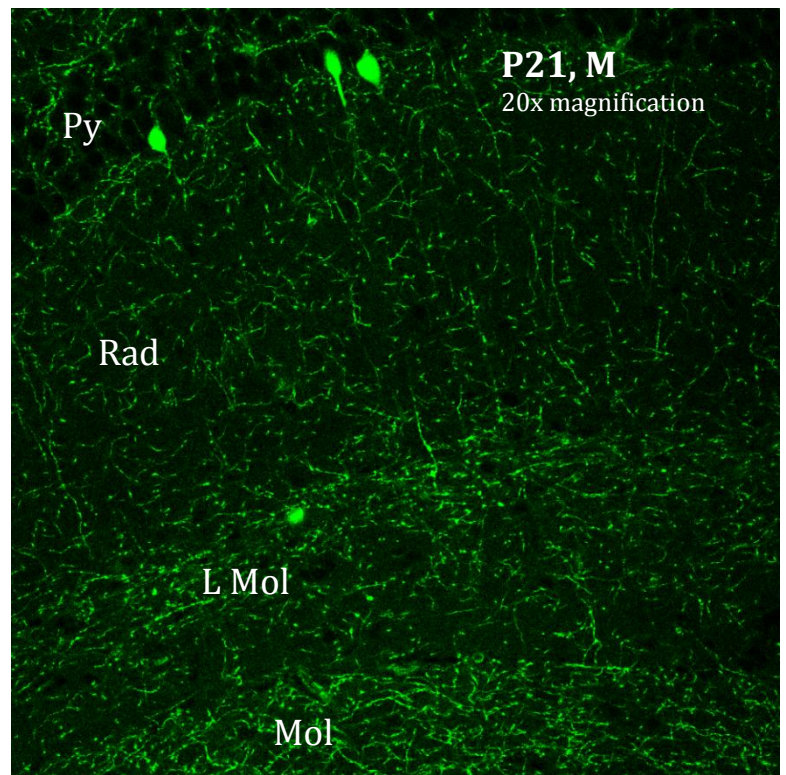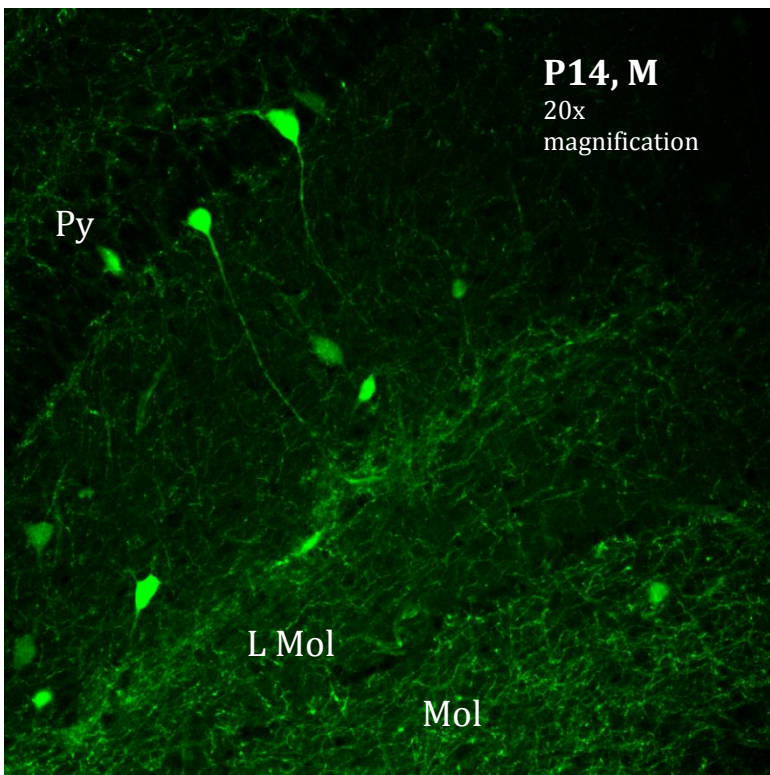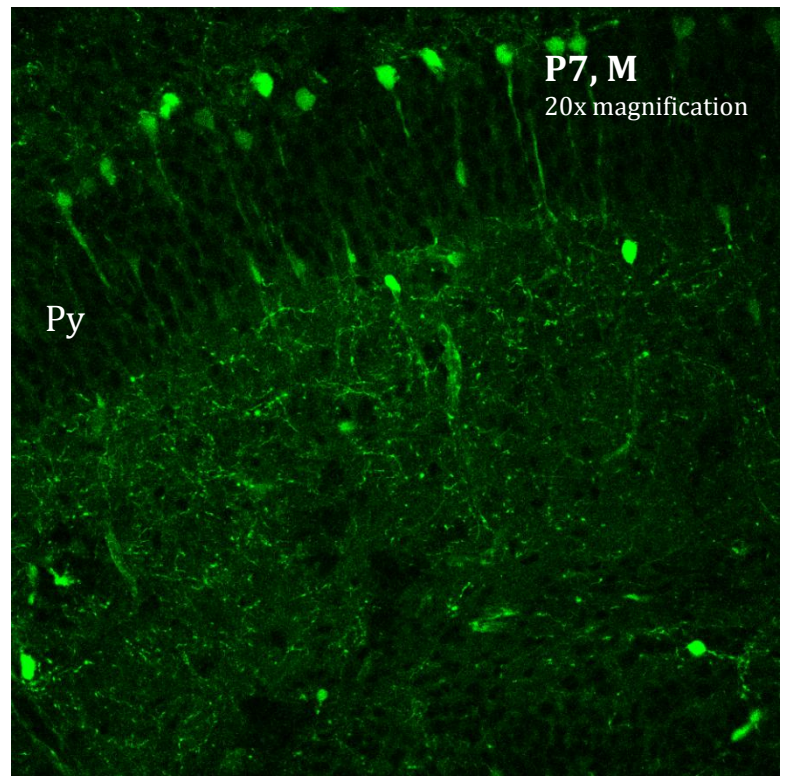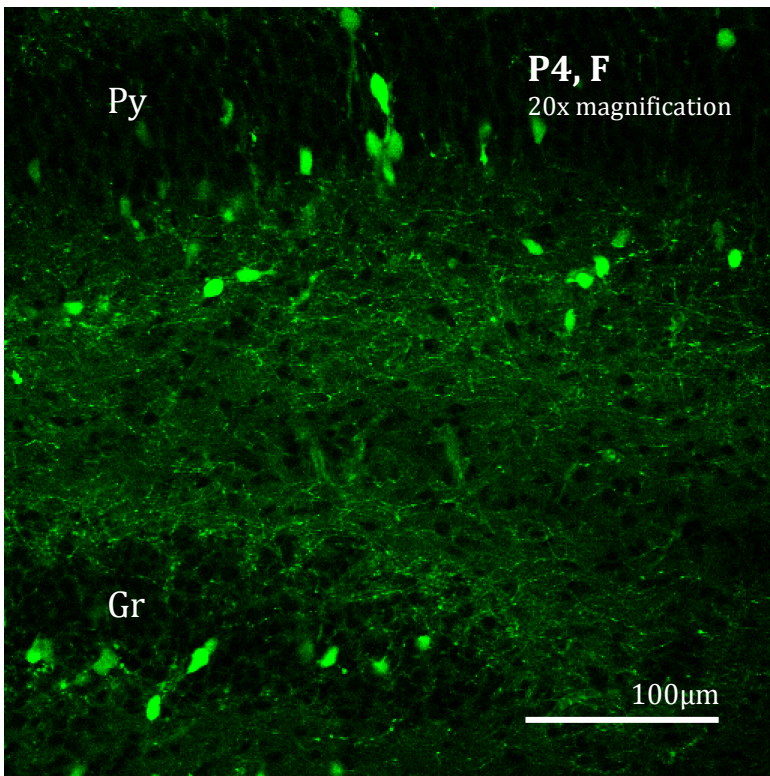
Figure 6a: *different layers in the CA1 region of the anterior hippocampus are shown from P4 to P21 in a 20 fold magnification. Note how distinguishable the layers became when compare P4/P7 and P14. Cholinergic neurons are also intercalating between pyramidal neurons at P7.*
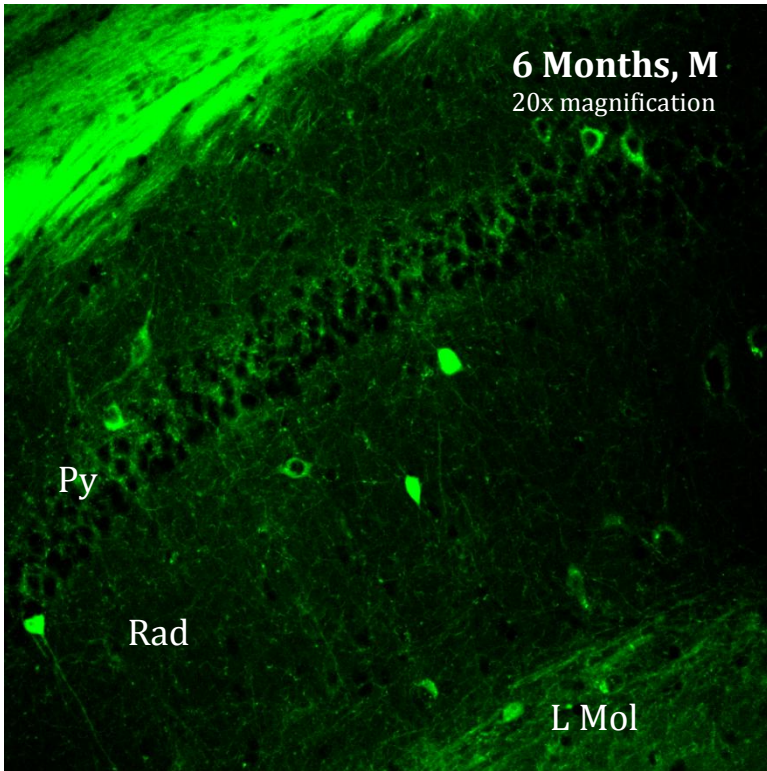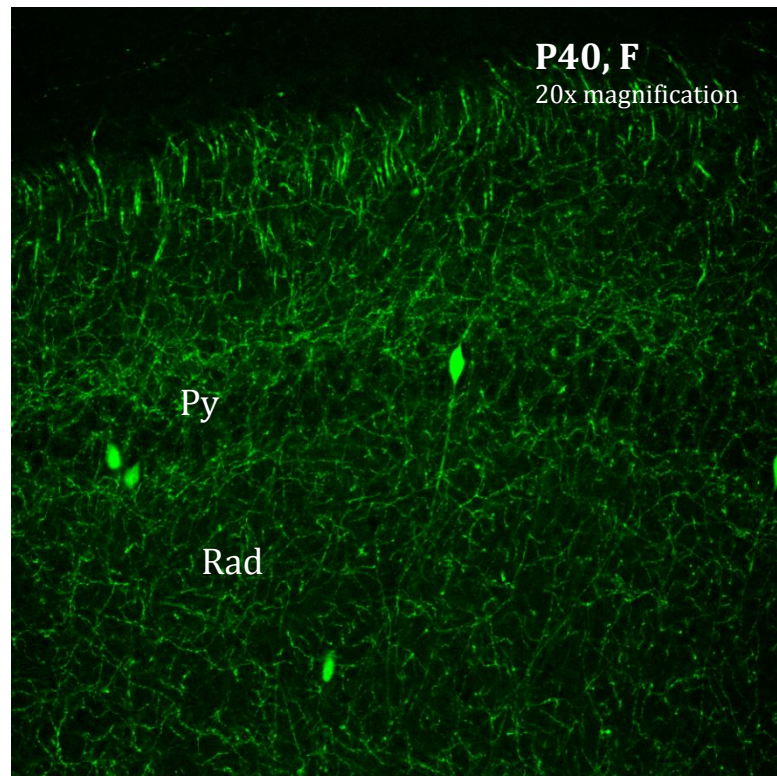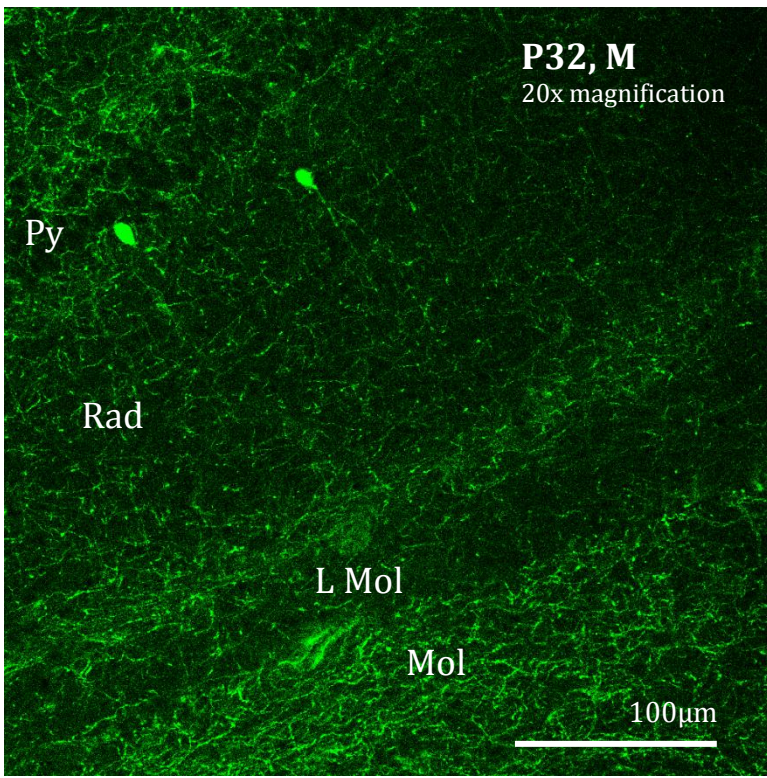
Figure 6b: *different layers in the CA1 region of the anterior hippocampus are shown from P32 to 6 months in a 20 fold magnification. Cholinergic neurons get more ovoid and the density of cholinergic fibers seems to decrease from P40 to 6 months old brains.*
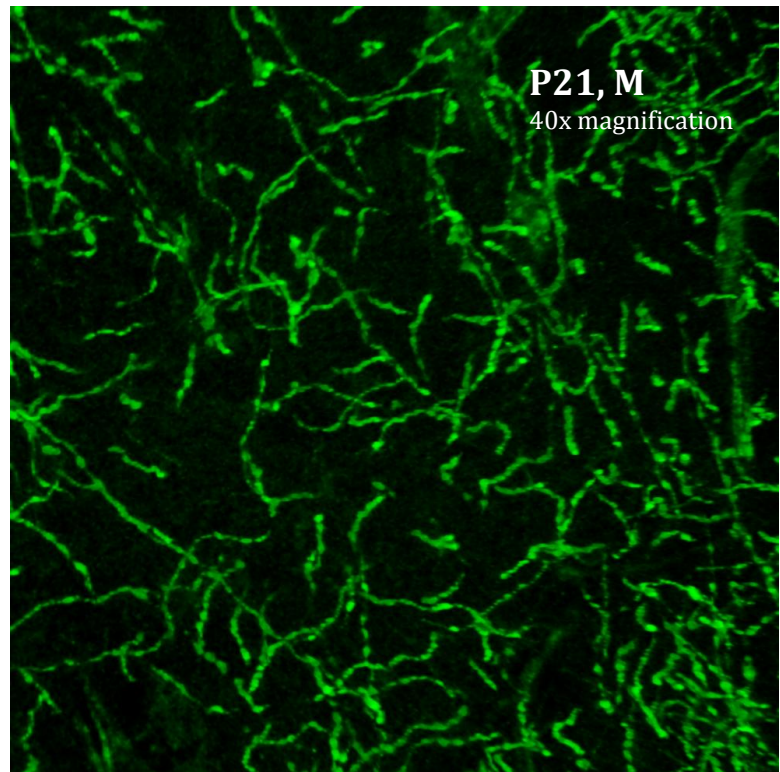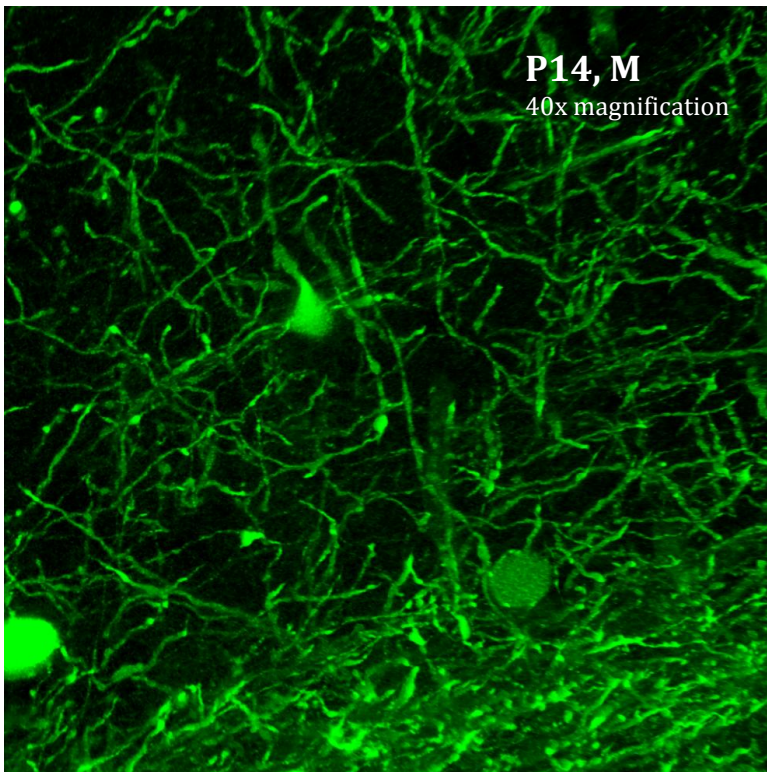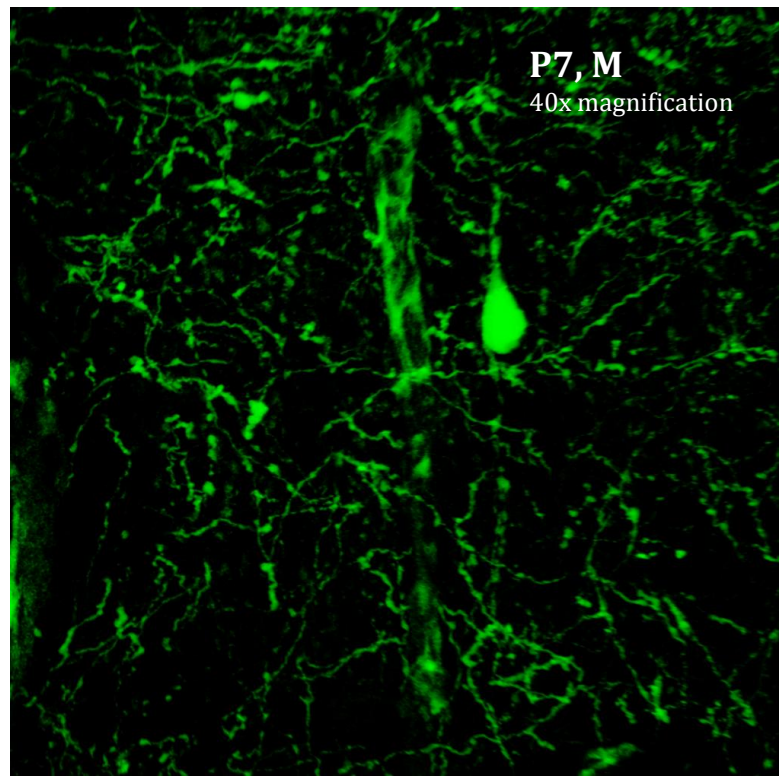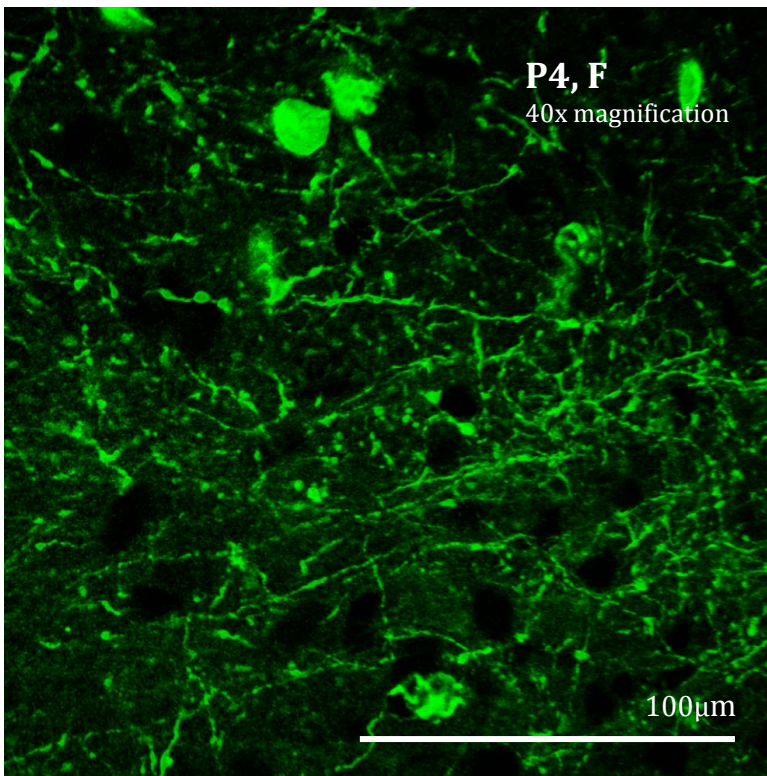
Figure 7a: *high magnification pictures (40 fold, z-stack with 11 slices and 1μm thick) of cholinergic fibers in the CA1 region of the anterior hippocampus from P4 to P21. Note how loose and disconnected the fibers are at young ages and how the fibers start to from a network.*
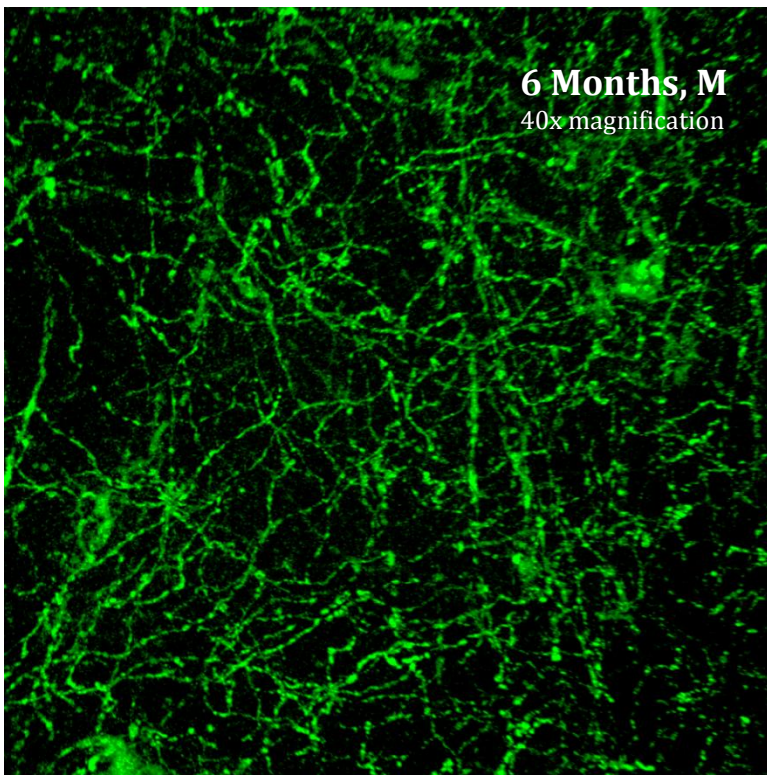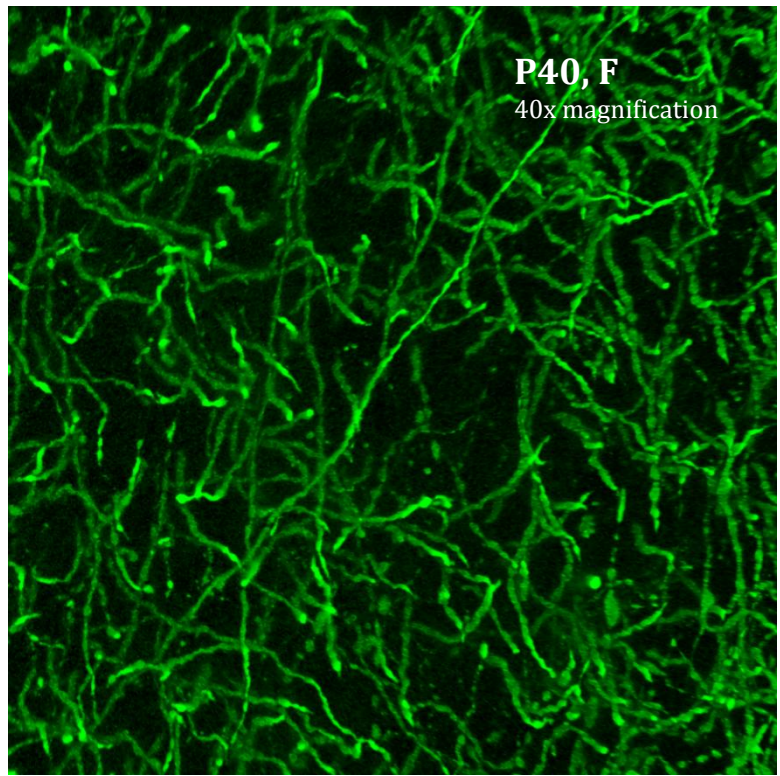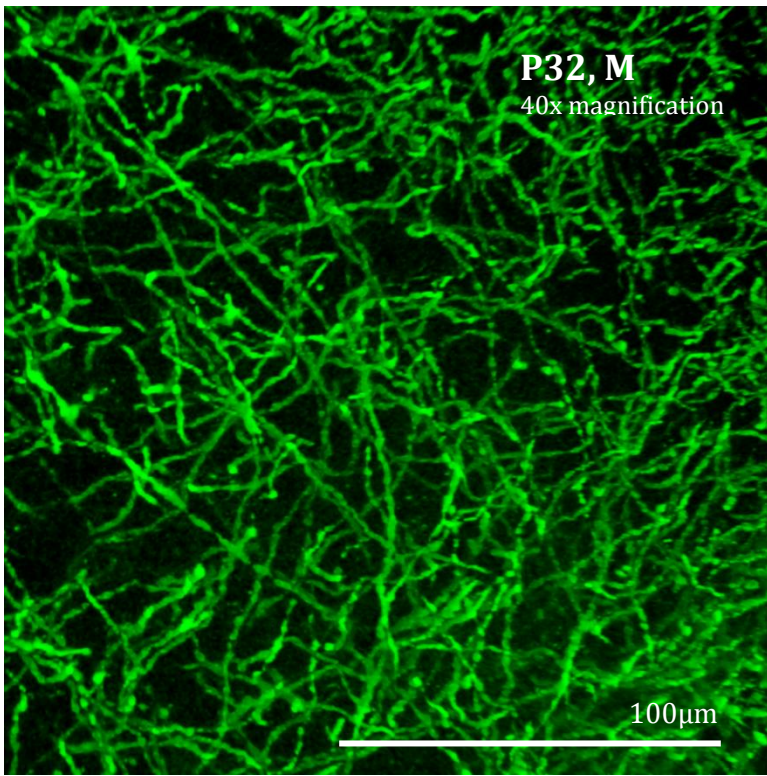
Figure 7b: *high magnification pictures (40 fold, z-stack with 11 slices and 1µm thick) of cholinergic fibers in the CA1 region of the anterior hippocampus from P32 to 6 months. The fibers get longer and continuous. Note how thinner the fibers appear from P40 to 6 months.*

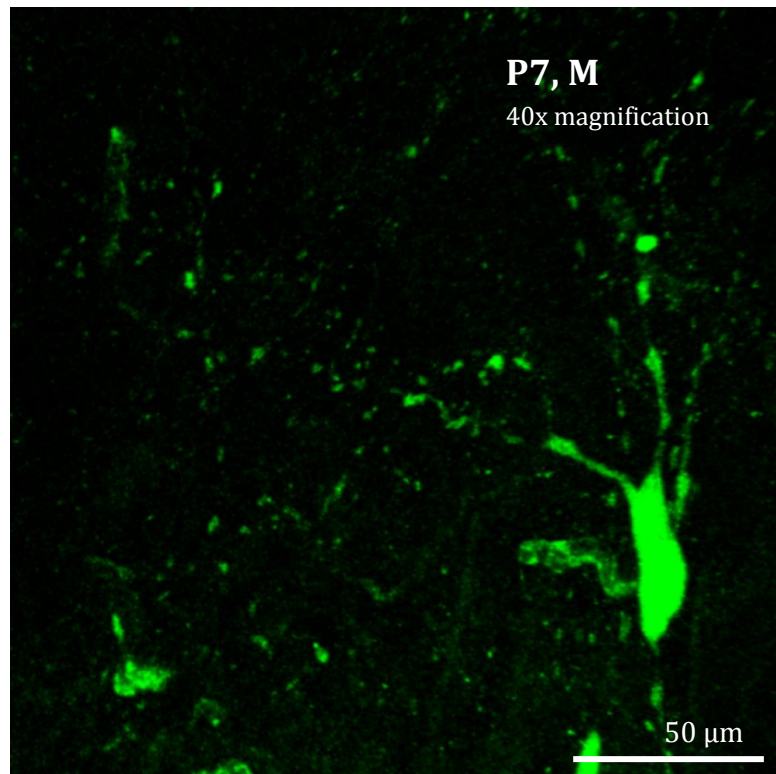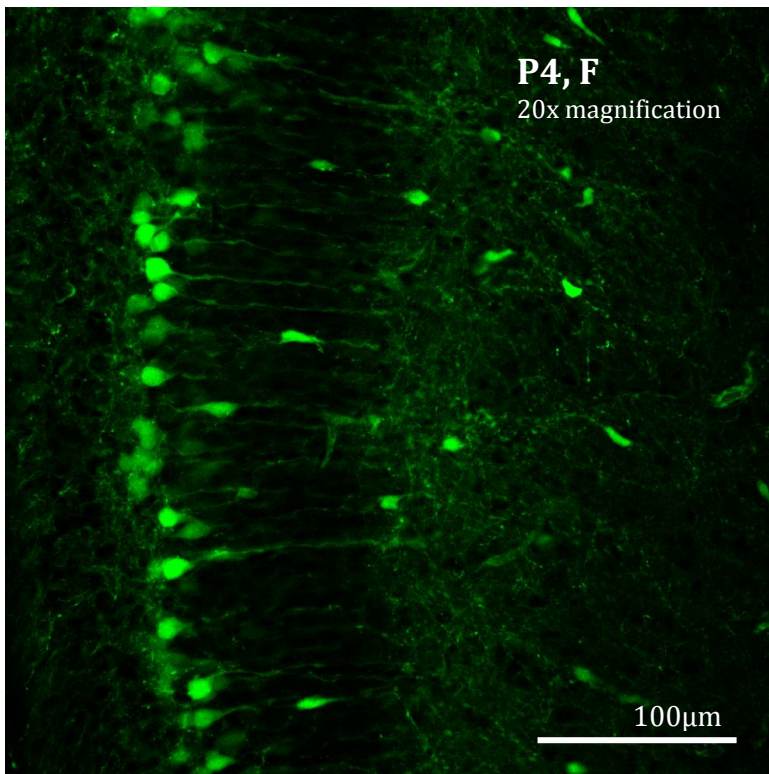**Figure 8**: <u>P4</u>: *cholinergic neurons intercalating between pyramidal cells in the pyramidal layer of the posterior hippocampus. <u>P7</u>: Polymorph and large cholinergic neuron is shown in the hilus of the DG of the anterior hippocampus.*
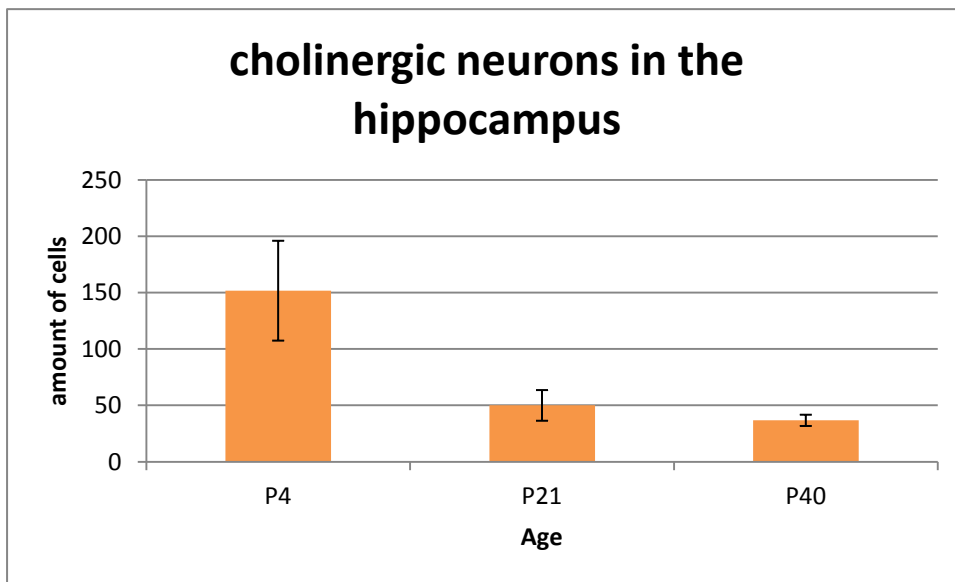
Graph 1: *Amount of cholinergic neurons found in the hippocampal formation of P4, P21 and P40. Mean and SD were calculated from 4 slices per age.*
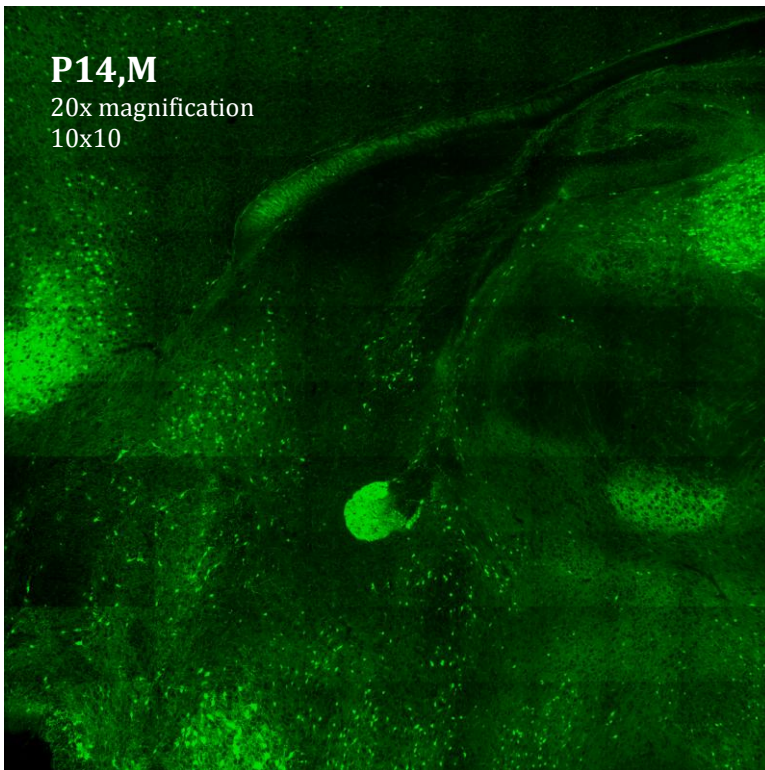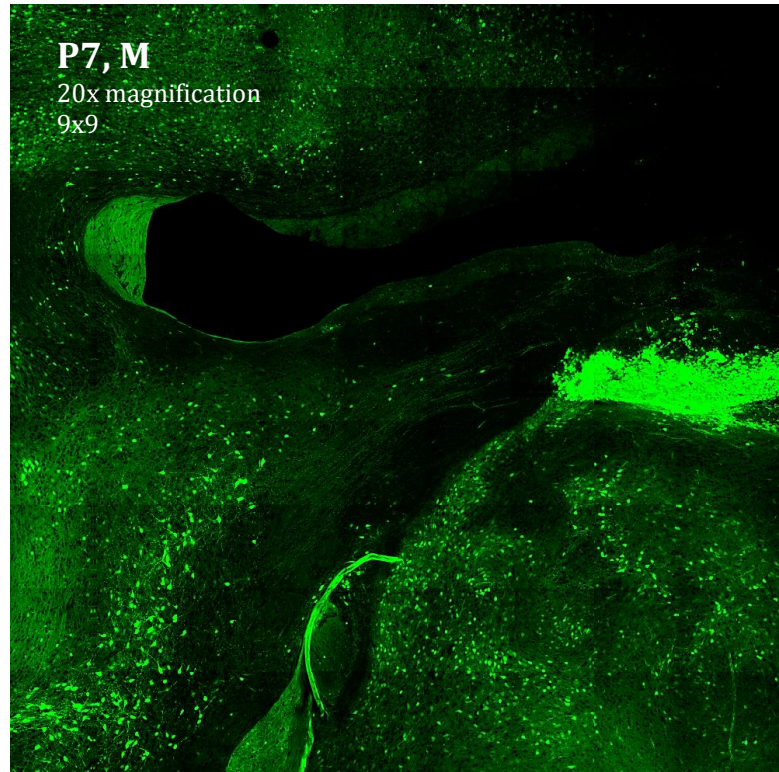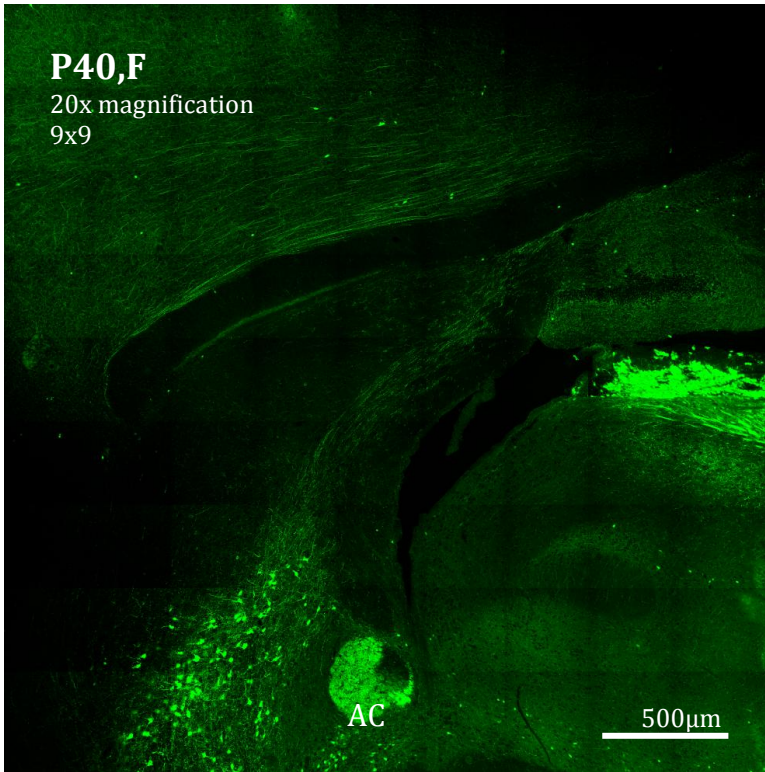
Figure 9: *Sagittal sections of the medial septum and hippocampal area in a 20 fold magnification. Note the cholinergic projection fibers to the hippocampal formation deriving from the medial septum and the lack of cholinergic fibers at one area in the anterior commissure. AC (anterior commissure)*

P21, M
10x magnification
CHAT stain
48hrs

100μm
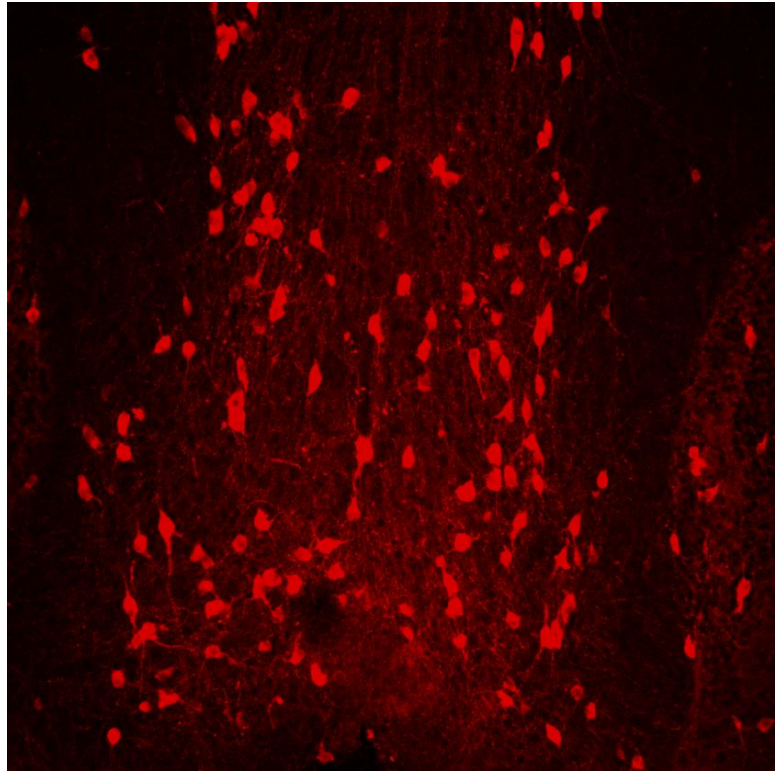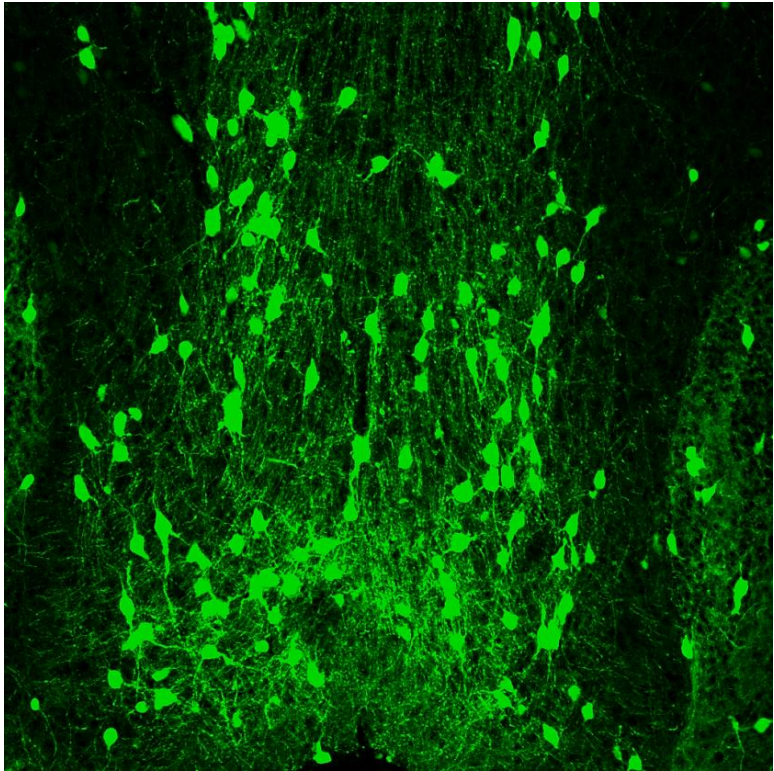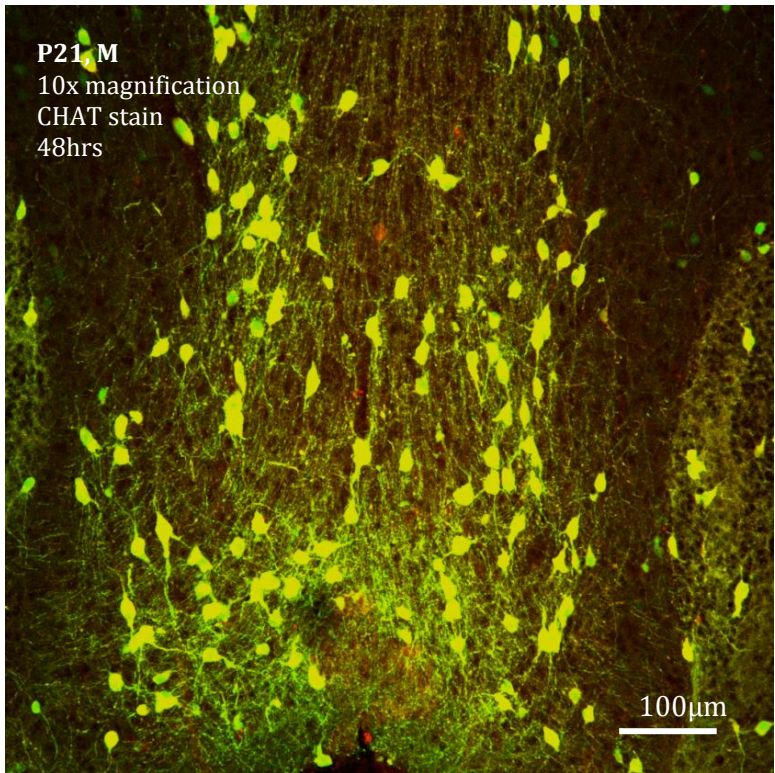
Figure 10a: ChAT immunohistochemistry in the medial septum. Confocal images taken with 488nm (GFP), 594nm (Alexa Fluor Red) and overlap is shown in the image above.
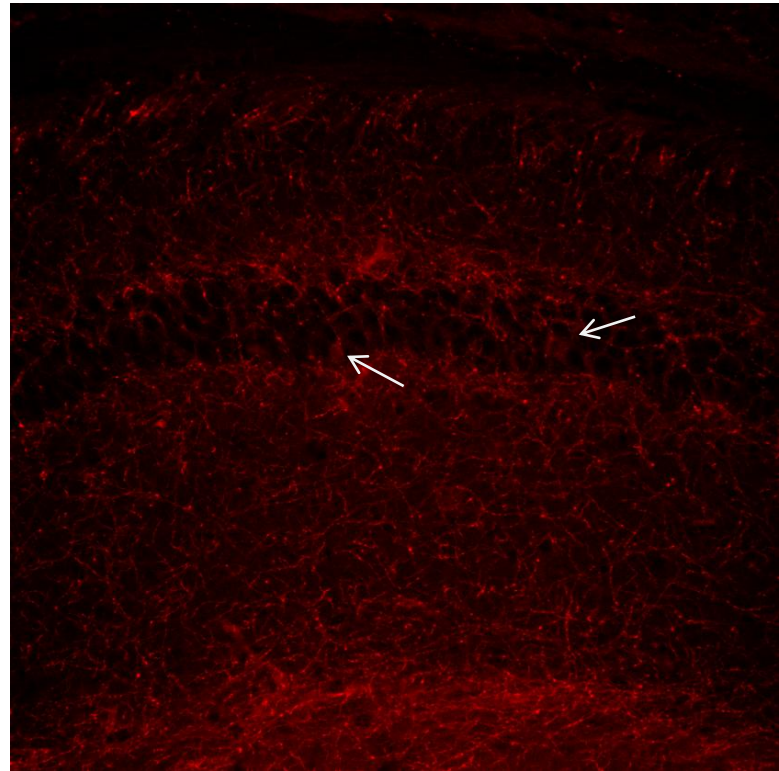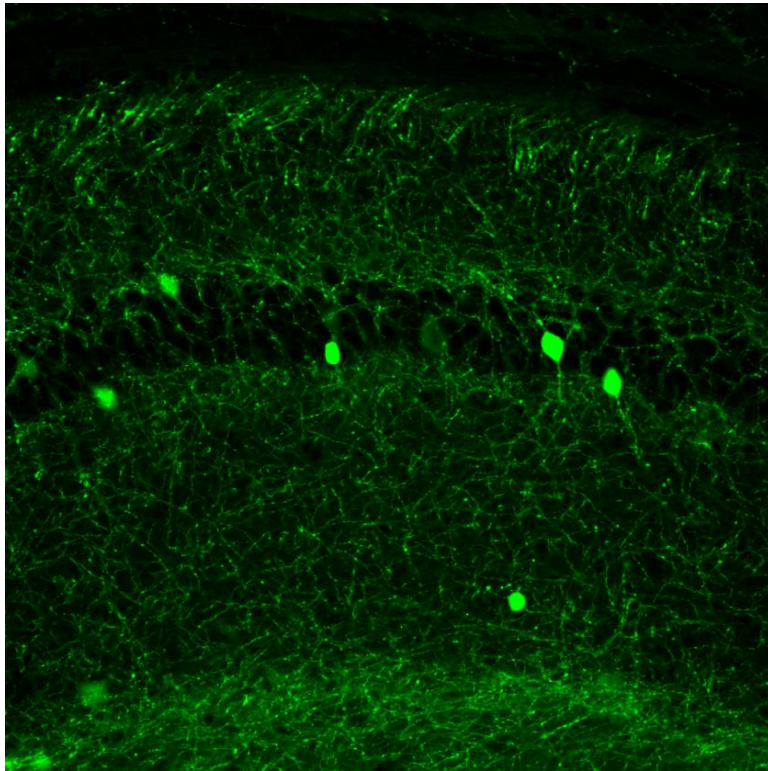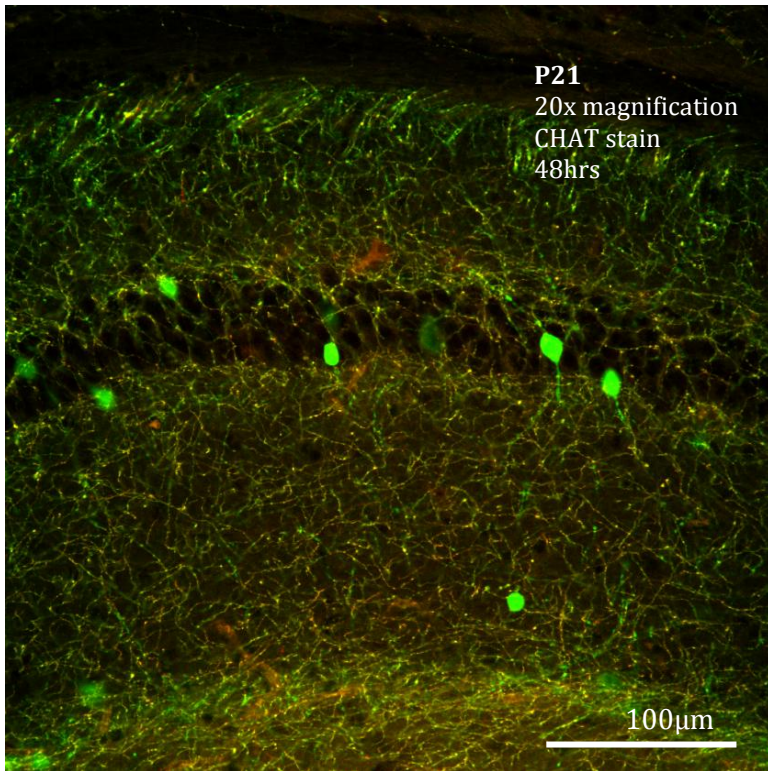
Figure 10b: CHAT immunohistochemistry in the CA1 region of the anterior hippocampus. Confocal images taken with 488nm (GFP), 594nm (Alexa Fluor Red) and overlap is shown in the image above. Note, fibers stained for CHAT antibody (red), but no cell somata are obtained. Arrowheads mark the position where a shadow of red stained cell bodies can be seen.

Discussion

The precise distribution and organization of cholinergic elements and the existence of cholinergic neurons in the hippocampal formation is still confusing and controversial (Clarke, 1985; Matthews et al., 1987; Frotscher et al., 1986; Fibiger, 1982; Wainer et al., 1985 and Blaker et al., 1988). The hippocampal formation has generally been considered to be lacking intrinsic cholinergic neurons since lesions to extrinsic hippocampal afferent systems lead to a major reduction in biochemically detectable ChAT and AChE-activity (Fibiger, 1982; Mellgren and Srebro, 1973; Storm-Mathisen, 1970 and Lewis et al., 1967). These studies also showed that removal of the septum or transection of the fimbria did not abolish all AChE and ChAT activity respectively in the hippocampus. This furthermore suggests that cholinergic neurons other than those in the medial septal nucleus/diagonal band complex contribute to the cholinergic innervation of the hippocampus.

In earlier studies ChAT immunoreactive neurons in the hippocampus were not observed (Kimura et al., 1980; Sofroniew et al., 1982; Armstrong et al., 1983). However, a small number of ChAT-positive neurons were noticed in the hippocampal formation in later survey studies (Wainer et al., 1985; Frotscher et al., 1986; Matthews et al.,1987; Blaker et al., 1988 and Kanaya-Ida and Ben Ari, 1989). With the exception of Blaker et al., 1988 all previous investigations were carried out by using monoclonal antibodies against ChAT in the adult and developing (Kanaya-Ida and Ben Ari, 1989) rat hippocampal formation. Frotscher et al. (1986), Matthews et al. (1987), Wainer et al. (1985) and Blaker et al. (1988) reported small, round to ovoid ChAT positive neurons in the adult hippocampus. Cholinergic neurons were found in almost all hippocampal layers although their distribution differs from the results obtained in this study. However, it is noteworthy that the hippocampal distribution of cholinergic neurons reported in those earlier studies varies between different research groups. For example, Wainer et al. (1985) found ChAT positive neurons mainly in the supra—and infragranular layer of the fascia dentata. Some neurons were observed in the supra-and infrapyramidal layer near the hilus of the DG and most of the positive cells were located in regio inferior. On the contrary, Matthews et al. (1987), Frotscher et al. (1986) and Blaker et al. (1988) discovered most of the cells in regio superior of the hippocampus, especially in the stratum lacunosum moleculare, the border of stratum lacunosum moleculare and stratum radiatum and the molecular layer of the DG. Matthews et al. (1987) observed 60% of the total ChAT positive neurons in region superior and of these three quarters in stratum lacunosum moleculare, especially near the border to stratum radiatum. Thirty persect of all observed ChAT positive neurons were also found in the DG, and there mostly in the molecular layer of the DG. The differences in cell distribution between this study and others could arise from the different

species that were compared (rats vs mice) and different applications (antibody concentration and incubation times) of immunohistochemical techniques amongst other research groups. Also the possibility remains, that ChAT antibodies don't detect the choline acetyltransferase itself, rather a protein with similar epitopes. This statement would imply, that all the results from the aforementioned authors could be questioned.

However, perhaps the most interesting result emerging from this project is the high number of cholinergic neurons in young murine hippocampus and a significant decrease in their abundance during adolescence and maturation. This is a qualitative conclusion that could not be verified with quantitative statistical analysis as we had only 1 subject per developmental stage. But nevertheless, a substantial decrease in the number of cholinergic cells in the hippocampus was seen from P4 to P21 (an average of 151 at P4 and 37 at P40). However, Kanaya-Ida and Ben Ari (1989) discovered an increase in the number ChAT positive neurons in the rat dentate gyrus between P5 and P20, and afterwards a decline until adult age. Our result is not consistent with this report. The difference of species (rat vs mice) together with the possibility of a non-specific antibody, which might pick up a similar epitope in an unknown protein, may explain the difference between the results in this study and the one by Kanaya-Ida and Ben Ari (1989).

Since the existence of intrinsic hippocampal cholinergic neurons is controversial, immunohistochemistry with anti-ChAT, anti- VACHT and anti-CHT antibody was performed. The neurons in the medial septum are widely considered to be cholinergic and were therefore selected as positive controls. All medial septum controls stained for ChAT, VACHT and CHT antibodies, but in the VACHT and CHT stained sections no positive fibers could be detected in the hippocampus. However, VACHT and CHT staining was very weak and even with longer incubation periods (48hrs) and higher antibody concentrations (1:20), no fibers could be seen. This is possibly the result of the use of an old antibody. The application of ChAT antibody stained cells in the medial septum as well as fibers in the hippocampal formation. Nevertheless, no somata could be observed in the hippocampus using this ChAT antibody. The aforementioned studies applied the ChAT antibody at a very high dilution (up to 1:9, comparing to the concentration in this study 1:20) and incubated up to 4 days. There might be a weak staining of cholinergic neurons in the hippocampus (figure 10b, arrowheads) but the presence of cholinergic neurons could not be confirmed by using this antibody. A reason for the non-appearance of ChAT positive cells could be the use of a polyclonal antibody. In previous studies, except for Blaker et al. (1988) who applied a polyclonal anti-CHAT antibody, high titer monoclonal antibodies were used to detect ChAT positive cells in the hippocampus. Also, the hippocampal neurons might have different

cell membranes, which the antibody is not able to penetrate easily. Yet, this does not explain why eGFP positive cells were seen in the hippocampus through all sections and ages that are not detectable by the application of a polyclonal anti-ChAT antibody. The turnover of ChAT might be fast and its steady state levels low, so that ChAT is not easily detectable, as compared to the expressed eGFP that may turn over slowly and accumulate in the cell bodies. Another possibility could be the fact that the green cholinergic neurons might have lost their phenotype and therefore do not express ChAT anymore. But due to the possibility that the eGFP could accumulate in the cells, the 488nm laser would be still able to capture it. An alternative idea is that in the CHATeGFP+/+ mice, eGFP expression might not be regulated exactly as that of ChAT (a "leaky promotor") – enabling an uncontrolled expression of GFP in some cells that are not cholinergic. It is important to note that bona fide cholinergic cells express eGFP (e.g. medial septum, cortex and striatum) in all tissue sections examined.

Ypsilanti et al. (2008) investigated the length of hippocampal cholinergic fibers during aging in 3 months and 24 months old rat brains. In older animals the length of cholinergic fibers is reduced, but no significant decreases in ChAT activity or ChAT protein levels in the hippocampus were observed in aged animals. Also, aging did not significantly alter the number and size of septal cholinergic cell bodies. These results would suggest that early stages of degeneration during normal aging impairs synapses and cholinergic axons of the hippocampus but not the production of cholinergic-specific proteins. The reduction of fiber density and thickness from P40 to 6 months in this report is in agreement with the results of Ypsilanti et al. (2008) that cholinergic fibers degenerate during aging. Also Geula and Mesulam (1989) detected a modest age-related decline of cortical cholinergic fiber density human postmortem brains. Noteworthy is also the perceived effect of quality loss of tissue that has already been mounted for a long time. This could also contribute to the observed loss of thickness from P40 to 6 months, because the older animals have already been mounted a few weeks before imaging.

The review of Bruel-Jungerman et al. (2011) pointed out that ACh might regulate hippocampal neurogenesis by having a pro-survival effect on the immature neurons to prevent them from dying and positively regulates the proliferation of hippocampal progenitor cells. During development, ACh might be critical for the improvement of cortical connectivity acting as a trophic factor to regulate the development and the morphology of cortical neurons. A loss of cortical cholinergic fibers in all layers occurs after cholinergic neuron-specific immunotoxicin injections at P0 and P2, which prevented basal forebrain afferents from projecting in the cortex. Application of AChE inhibitors enhanced the survival of newborn hippocampal neurons. Altogether, ACh may regulate several steps of hippocampal

neurogenesis and promote both the integration and survival of the immature neurons in the neuronal circuitry and the proliferation of progenitors. Hippocampal-dependent learning and memory are influenced by the hippocampal cholinergic system and both loss and gain of cholinergic balance causes learning impairment. These conclusions in the review of Bruel-Jungerman et al. (2011) suggest that ACh could act as a nursing factor for developing neurons in the hippocampal formation. Furthermore, hippocampal eGFP expressing cholinergic neurons in this study may switch their neurotransmitter phenotype after functioning as "supporting neurons" during development. This could explain the decrease of cholinergic cell numbers in older animals, as most of neurogenesis takes place at young ages.

To confirm the cholinergic phenotype of the eGFP expressing neurons in the hippocampus, antibodies corresponding to the ones used in the studies of Frotscher et al. (1986), Matthews et al. (1987), Wainer et al. (1985), Blaker et al. (1988) and Kanaya-Ida and Ben Ari, (1989) should be used to detect ChAT containing cells. Also staining for other neurotransmitter or their processing and modifying enzymes, e.g. GABA or serotonin, should be performed to exclude or confirm a phenotypical switch of cholinergic neurons. For statistical significance more animals per each age would be needed. Nevertheless, these results could provide a new view of the development of cholinergic neurons and a proof of the existence of this type of neurons in the hippocampal formation Moreover, the CHATeGFP reporter mice are a suitable strain to do further investigations on cholinergic neurons in this part of the brain.

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Boston University

School of Medicine
Department of Pathology and
Laboratory Medicine

Jan Krzysztof Blusztajn, Ph.D
Professor

Department of Pathology and Laboratory Medicine
Medical Campus
72 East Concord Street,
L808
Boston, Massachusetts 02118
jbluszta@bu.edu

May 15, 2013

To Austrian Marshall Plan Foundation:

Re: Approval of Report

I am writing to confirm, that the present report about "Hippocampal Cholinergic Neurons in developing CHATeGFP Reporter Mice", composed by Jasmine Rinnofner in the laboratory of cognitive neuroscience in the Department of Pathology at Boston University is correct as regards content.

Please don't hesitate to contact me if further information is required.

Sincerely

Jan Krzysztof Blusztajn, Ph.D.
Principal Investigator