Fall 2019

Neural control of facial sweat gland secretion in horses

Lu Liu
lliu1@iastate.edu

Neural control of facial sweat gland secretion in horses

Lu Liu

2019.11.26

BMS 599 Creative Component

Biomedical Sciences Department

Iowa State University

Abstract:

For sweat glands, it has been shown that sympathetic neurons express a cholinergic/noradrenergic co-phenotype in their innervation in the trunk of mice.(Schütz et al. 2008). It is unknown if facial sweat glands are innervated the same way. Gustatory sweating is an abnormal sweating condition. People can sweat profusely when eating or drinking, or even thinking about eating or drinking. We hypothesize that the facial sweat glands are controlled differently and can be related to parasympathetic neurons from cranial nerves. Some samples from human donors have been tested, but few sweat glands can be targeted since the donors were older people. Thus, it was decided to use horse tissue for this project to obtain greater numbers of sweat glands on the face. A challenge for this project included a lack of availability of an equine-specific antibody to label vesicular ACh transporter. A successful antibody for tyrosine hydroxylase was identified for horse tissue but the staining results suggest a lack of specificity for tyrosine hydroxylase. This demonstrates a need for a more specific antibody in horse to determine actual labeling of tyrosine hydroxylase. In addition, increase numbers of samples and slide preparations may improve the ability to identify properly label tyrosine hydroxylase.

Introduction:

A sweat gland is a type of exocrine gland which produces and secretes substances above epithelial surface through a duct. There are two types of sweat glands-Eccrine sweat glands and apocrine glands and human have both types. Eccrine sweat glands are distributing all over the body. They produce a water-based secretion, which can cool the body. Apocrine sweat glands are mostly located in axilla and perianal areas in humans. (Hu et al. 2018) There are effective sweat glands in hoofed animals, such as the horses, donkeys and camels. For horses, most sweat glands open into the hair follicle which belong to the apocrine glands. However, they are similar to human's eccrine sweat glands as they secrete a great quantity of watery fluid.(Lovatt Evans et al. 1957) From previous research, it is known that sweat glands on the trunk are innervated by sympathetic nerves producing acetylcholine(ACh) rather than parasympathetic nerves in human body.(Habecker et al. 1997) It is unknown if that condition is the same in facial tissue. Gustatory sweating which is also called Frey syndrome or auricultotemporal syndrome, is an abnormal sweating condition. People can sweat profusely when eating or drinking, or even thinking about eating or drinking. (Licht and Pilegaard 2006) This neurological abnormality is derived from misdirected regeneration of parasympathetic fibers normally innervating salivary glands onto sweat glands following injury to the auriculotemporal nerve. Auriculotemporal nerve is a branch of the mandibular nerve(V3), which is the largest divisions of the trigeminal nerve(CN V). Auriculotemporal nerve also delivers postganglionic parasympathetic nerves from the glossopharyngeal(CN IX) nerve to the parotid gland. Thus, there is pathological sweating in the preauricular area on the side of the injury to the nerve. Based on this disorder, it is shown that misdirected regeneration of the auriculotemporal nerve lead to the disease, we hypothesize that it is likely that the facial sweat glands can be controlled by the parasympathetic fibers directly, may be another cranial nerve like facial nerve(CN VII), without the help from sympathetic fibers. Thus, the purpose of this project is to explore out whether facial sweat glands are innervated by sympathetic nerves or parasympathetic nerves.

Background:

In humans, there are two major sweat gland types, eccrine and apocrine. 90% of the total number of sweat glands is eccrine sweat glands. Apocrine sweat glands mainly locates in the axillae and external auditory meatus. The basic structure of eccrine sweat gland is made of tubular epithelia which comprise the secretory coil and a reabsorptive sweat duct(RSD). RSD comprises a straight segment and the intraepidermal sweat duct, which opens onto the skin surface. Apocrine glands are composed of a glomerulus of secretory tubules that funnel towards an excretory duct that opens into the hair follicle. The sweat gland of the horse is a tubular coil gland opening into the hair follicle. The secretory portion of the glands set a compact and large glomerulus in the perineal region. In this area, one can easily observe with a magnifying lens.

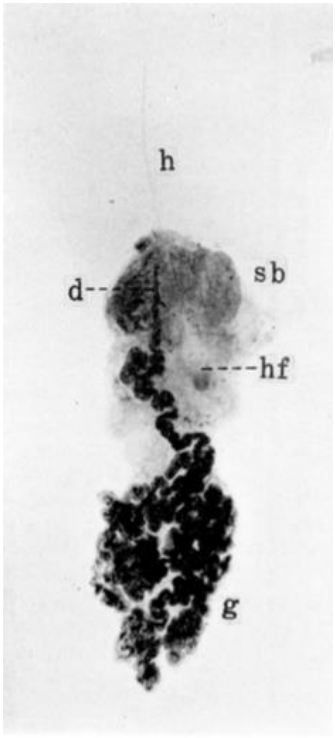


Fig 1. The sweat gland from the perineal region, being dissected out with the associated pilosebaceous system. The slide was stained with acetocarmine. Magnification: ×15. Abbreviations: d-sweat duct, hf-hair follicle, g-sweat glomerulus, h-hair, sb-sebaceous gland.

glomeruli in the corium on a vertical cut of the skin. The secretory tubule of the glands consists of a layer of gland cells and a myoepithelium and rests on a basement

membrane of connective tissue. The excretory duct is constituted by an inner layer of tall cells and an outer layer of low cuboidal cells. (Takagi and Tagawa n.d.)

The secretion of sweat is stimulated by ACh through sympathetic nerves rather than parasympathetic nerves. During development, the sympathetic innervation of sweat glands switches from noradrenergic to cholinergic control. (Schütz et al. 2008) The transformation is based on a ligand for the LIFRBeta-gp130 receptor. Cytokines act through the gp130 receptor which is present in sweat glands. Selective elimination of gp130 receptor in sympathetic neurons prevents the acquisition of cholinergic features without influencing other properties of sweat gland innervation. Gp130-signaling has an essential role in the target-dependent specification of the cholinergic neurotransmitter phenotype (Stanke et al. n.d.). The functional switching of chemical transmission during tissue development is resulted from selecting the pre-existing cholinergic effector neurons (Habecker et al. 1997). To find out if the facial sweat glands are innervated by sympathetic nerves or parasympathetic nerves, a specific target should be detected.

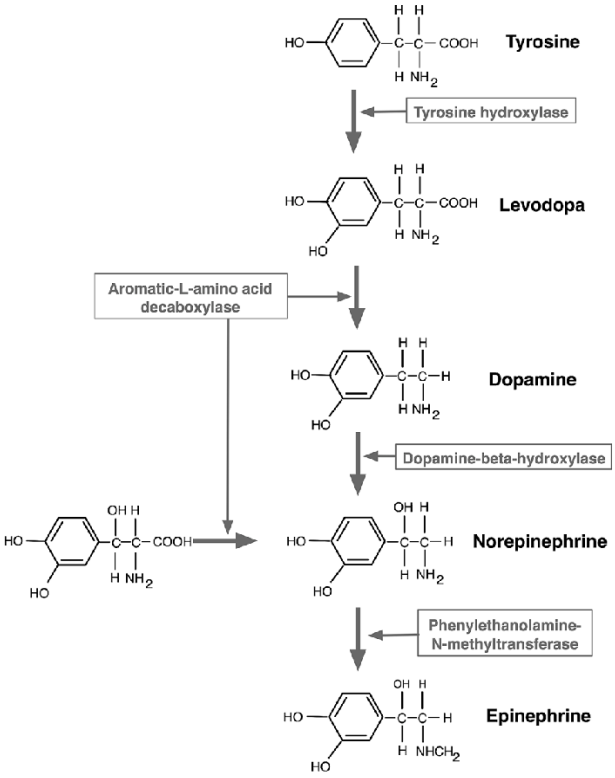


Fig 2. Catecholamine synthetic pathway. L-tyrosine is converted to L-DOPA by tyrosine hydroxylase. L-DOPA can then be converted to dopamine by aromatic L-amino acid decarboxylase, which in turn can be converted to norepinephrine by dopamine beta-hydroxylase. Lastly, norepinephrine can be converted to epinephrine by the enzyme phenylethanolamine N-methyltransferase. (Kaufmann 2008)

Sympathetic neurons express a cholinergic/noradrenergic co-phenotype in sweat gland innervation in mice.(Schütz et al. 2008). The vesicular ACh transporter (VAChT) has been used cas a marker of cholinergic nerves while tyrosine hydroxylase (TH is the catecholamine synthesis ' rate-limiting enzyme. It catalyzes the formation of L-DOPA. The components of the pathway are dopamine, norepinephrine and epinephrine, essentially as neurotransmitters and hormones in the central and peripheral nervous systems. In the latter, they are synthesized in the adrenal medulla. These catecholamines play important roles in many brain functions, such as attention, memory, cognition, and emotion. As the hormone responsible for the fight-or-flight response, many tissues throughout the body are affected by epinephrine. Therefore, catecholamine abnormalities have many repercussions, including hypo- and hypertension, bipolar disorder, addiction, and dystonias. Because of this, TH's activity as the pathway enzyme is of great interest in many biomedical research fields (Daubner, Le, and Wang 2011) For this project, TH can be used to detect noradrenergic fibers.

VAChT works on loading ACh into secretory vesicles in cholinergic neurons that allows ACh to be secreted.(Arvidsson et al. n.d.) VAChT relies on an exchange between protons that were first pumped into the vesicle diffusing out, which act as an antiporter. Then ACh is carried into the vesicle by exiting protons. The transport of Ach utilizes a proton gradient set by a vacuolar ATPase. VAChT has been shown to be useful as a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. In the peripheral nervous system, abundant distribution of VAChT-positive neurons can be detected with the predicted distribution of cholinergic neurons in the autonomic nervous system which innervate organs such as the eye, salivary, lacrimal glands, respiratory tract, reproductive organs (Weihe et al. 1996).

Both TH and VAChT immunoreactivities are present in sympathetic nerve fibers around trunk sweat glands, while only VAChT can be seen in parasympathetic nerve fibers., In species? after five days of birth, the immunoreactivities are more obvious for both TH and VAChT around sweat glands in transgenic mice(Schütz et al. 2008).

As for face, it is possible that the facial sweat glands are innervated by parasympathetic nerves directly. Because, on the face, which is close to cranial nerves, can be innervated by cranial nerve directly, so that there may not have the noradrenergic sympathetic nerves. Therefore, we hypothesized that , on the facial tissue, there are only cholinergic neurons around sweat glands and no noradrenergic neurons. To prove/disprove this hypothesis, we use TH and VAChT immunohistochemistry in equine tissues to 1) confirm trunk sweat glands are innervated by sympathetic cholinergic nerves and 2) to determine whether facial sweat glands are innervated by parasympathetic cholinergic nerves.

Methods:

The project was originally designed for human subjects, but it was difficult to obtain the facial tissue from healthy adults. Some samples from old human donors have been tested, however very few sweat glands were found, it is likely they had lost many sweat glands as they get older. That was the reason that we used horses in the present study.

Horses were from necropsy lab in the Department of Veterinary Pathology, Iowa State University. Eight sites of facial tissue and one site on the trunk are collected, in order to compare their immunoreactivities. As shown in the picture, eight sites are midline forehead, 5 cm caudal to lateral canthus, midline between the eyes, central masseter, rostral end of facial crest, lateral lip, ventral intermandibular and base of the ear. These eight sites have the highest possibility to locate high density of sweat glands on horses' face. Tissues from trunk area were also collected as a comparison with facial tissues.

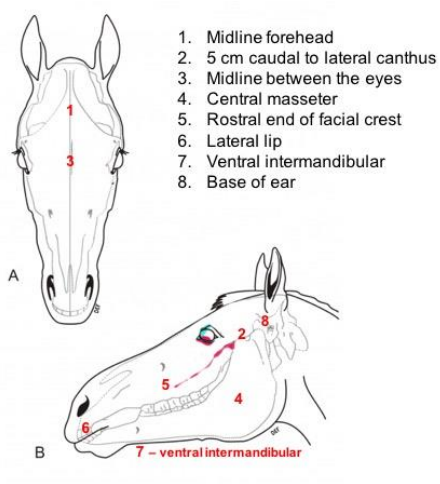


Fig 3. Eight sites on equine facial skin collected for the detection of sweat glands.

Immunohistochemical(IHC) detection of antigen on the tissue section represents a major technological need in research and clinical diagnostics. Green fluorescent protein has been a scientific breakthrough for cell biology predating 1960. (Remington 2011) Fluorescent detection is commonly used to visualize antigens. The fluorochrome can be conjugated either directly to the primary or secondary antibody or to streptavidin. (Zhang et al. 2017) Immunofluorescence methods have been used for the detection of tyrosine hydroxylase.

Paraffin-embedded sections mounted on microscopic slides were deparaffinized with xylene (2*2 min), rehydrated through a graded series of ethanol(1*2 min each). Unspecific antibody binding was blocked by incubation in 2% bovine serum albumin (BSA), triton X in PBS for 60 min in room temperature and incubated overnight with TH (concentration 1:1000). After 6*10 min wash in PBS, sections were incubated with secondary antibody (Alex Fluor 488) for 60 min in room temperature. Then slides were washed in PBS for 5*5 min. Hoecht nuclear counterstain (1:5000) was applied on the slides for 15-20 min. Then the slides were washed in PBS for 5*5 minutes and were dried in room temperature overnight. They were dehydrated through ehtanol series in a graded series. They were covered with resin mounting media under coverslip (held in xylene while coverslipping).

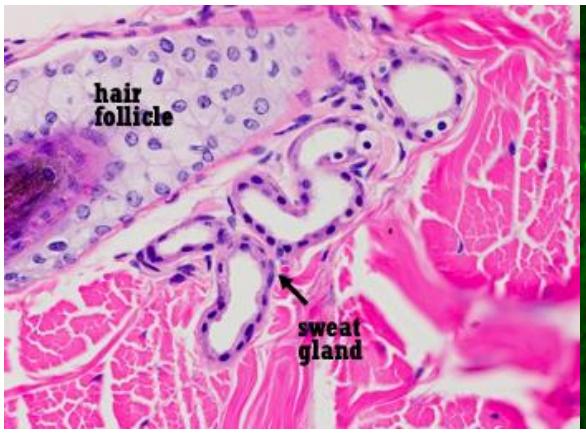
To test if the TH antibody(#AB152, millipore) can work for horses, adrenal medulla was first used. Subsequently,, skin tissues from face and were collected to detect the condition of TH.

In Dr. Kanthasamy's lab, they have plenty successful experience with TH immunofluorescent methods. (Harischandra et al. 2019) However, for VAChT, since lots of auto-fluorescent has been found on the tissue specimens, horse-radish peroxidase (HRP)-linked secondary antibody was used for immunohistochemistry. This is a rapid and highly sensitive method for the detection of VAChT in horse skin tissues.

Paraffin-embedded sections mounted on microscopic slides were deparaffinized with xylene, rehydrated through a graded series of ethanol. Antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) in steamer for 20-30 minutes. Then the sections were incubated in 3% H_2O_2 for endogenous peroxidase block for 20 min. Unspecific antibody binding was blocked by incubating the slides in 3% BSA, 3% normal serum and 0.05% Triton-x 100 in TBS for 30 min. The sections were Incubated overnight with VAChT, and different concentrations had been tried to test the antibody. The concentration of 1:500 and 1:1000 was used for mouse small intestine. And the concentration of 1:100 and 1:500 has been tested for horses tissue. After 4*5 min of wash in TBST, sections were incubated with HRP polymer reagent for 30 min. Then they were incubated with chromogen solution for 5 min, and the change of red color was observed at the same time to ensure the color was not too strong. Then slides were washed in distilled water and counter stain in Mayer's Hematoxylin was applied for 1.5 min. Slides were washed in cold running tap water for 5 min. Dehydration through ethanol series in a graded series was applied for 5 min each. Then covered with resin mounting media under coverslips(hold in xylene while coverslipping).

VAChT(#ABN100 millipore sigma; #139 103 Synaptic Systems) from two companies were used for the present study. They were first tested in mouse and horse small intestine tissues and were then used in face and trunk skin tissues.

Results:



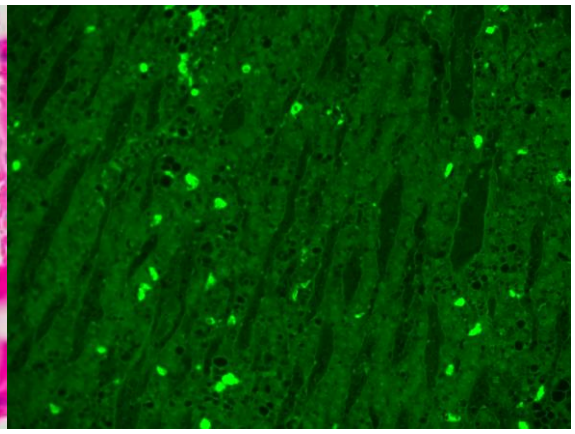
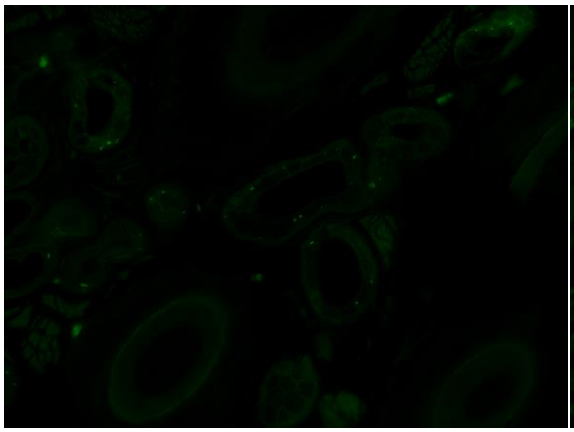


Fig 4. Sweat gland in high magnification Fig 5. TH staining testing in horse adrenal medulla with positive results

From the eight sites of facial sweat glands, sites 2, 5, 8 can be observed with higher density of sweat glands.

For the testing of TH, we found clear positive results around horse adrenal glands. Thus, TH antibody can be used to detect TH in equine tissues.



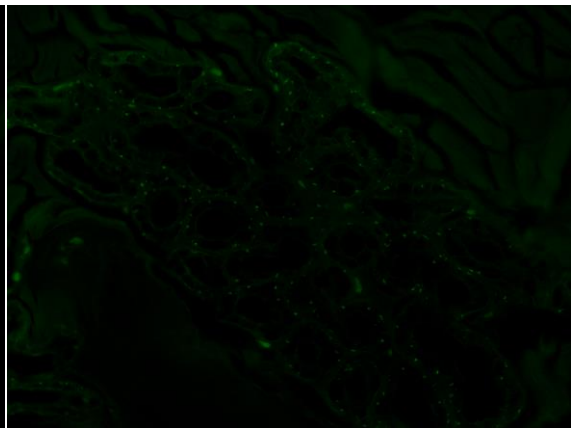


Fig 6 Trunk tissue with TH staining, negative result Fig 7. Facial tissue with TH staining, negative result

However, the following experiments with trunk and facial tissues have unexpected results. Few positive spots can be found under fluorescent microscope.

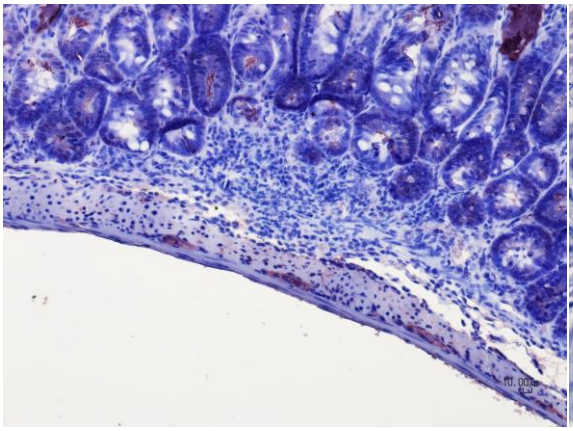
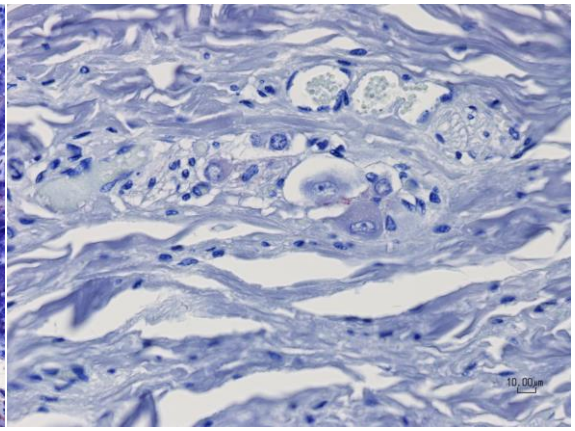



Fig 8. Mouse small intestine with VAChT staining, positive results Fig 9. Horse small intestine with VAChT staining, negative results

For the testing of VAChT, unfortunately, both antibodies we tried did not work for equine tissues. While positive results can be detected in the mouse small intestine in the concentration of 1:500. High concentration(1:100) for horses tissue can increase the existence of non-specific staining.

Discussion:

The results for TH staining are unexpected, since it is known that trunk sweat gland uses the sympathetic nerve to control sweat secretion, noradrenergic neurons should be detected around sweat glands. It is possible that the cut did not meet the neurons, and more slides can be tested.

However, for VAChT, since auto-fluorescence-caused false positive is a great concern, horse-radish peroxidase(HRP)-linked secondary antibody was used for immunohistochemistry.

Though BLAST has been used to check the DNA sequence of VAChT in both mouse and horse, they have more than 90% similarity, however, the antibodies were not able to detect the antigens on the horse sections. There is still species difference exist.

Since this is my Master creative component thesis, time is limited for the project. In the future, the primary antibody to recognize equine VAChT needs to be identified for this research. Number of sample was not enough in the present study to yield conclusive results in the identification of the nerve fibers. Although the present study did

not yield conclusive results, we are confident that with a proper primary antibody against equine VAChT and a number of tissue sections showing sweat glands and associated autonomic nerves, one shall be able to prove/disprove the hypothesis whether facial sweat glands are innervated by sympathetic or parasympathetic nerves.

References:

Arvidsson, Ulf, Maureen Riedl, Robert Elde, and Bjo Rn Meister. “Vesicular Acetylcholine Transporter (VAChT) Protein: A Novel and Unique Marker for Cholinergic Neurons in the Central and Peripheral Nervous Systems.” : 14.

Daubner, S. Colette, Tiffany Le, and Shanzhi Wang. 2011. “Tyrosine Hydroxylase and Regulation of Dopamine Synthesis.” *Archives of Biochemistry and Biophysics* 508(1): 1–12.

Habecker, B. A. et al. 1997. “A Sweat Gland-Derived Differentiation Activity Acts through Known Cytokine Signaling Pathways.” *The Journal of Biological Chemistry* 272(48): 30421–28.

Harischandra, Dilshan S. et al. 2019. “Enhanced Differentiation of Human Dopaminergic Neuronal Cell Model for Preclinical Translational Research in Parkinson’s Disease.” *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*: 165533.

Hu, Y., C. Converse, M. C. Lyons, and W. H. Hsu. 2018. “Neural Control of Sweat Secretion: A Review.” *The British Journal of Dermatology* 178(6): 1246–56.

Kaufmann, Horacio. 2008. “L-Dihydroxyphenylserine (Droxidopa): A New Therapy for Neurogenic Orthostatic Hypotension: The US Experience.” *Clinical Autonomic Research* 18(S1): 19–24.

Licht, Peter B., and Hans K. Pilegaard. 2006. “Gustatory Side Effects After Thoracoscopic Sympathectomy.” *The Annals of Thoracic Surgery* 81(3): 1043–47.

Lovatt Evans, C., A. Myfanwy Nisbet, and K.A. Ross. 1957. “A Histological Study of the Sweat Glands of Normal and Dry-Coated Horses.” *Journal of Comparative Pathology and Therapeutics* 67: 397-IN47.

Remington, S. James. 2011. “Green Fluorescent Protein: A Perspective.” *Protein Science* 20(9): 1509–19.

Schütz, B. et al. 2008. “Sweat Gland Innervation Is Pioneered by Sympathetic Neurons Expressing a Cholinergic/Noradrenergic Co-Phenotype in the Mouse.” *Neuroscience* 156(2): 310–18.

Stanke, Matthias et al. “Target-Dependent Specification of the Neurotransmitter Phenotype: Cholinergic Differentiation of Sympathetic Neurons Is Mediated in Vivo by Gp130 Signaling.” : 11.

Takagi, Shunzo, and Masayuki Tagawa. “A Cytological and Cytochemical Study of Sweat Gland of the Horse.Pdf.”

Weihe, E. et al. 1996. “Visualization of the Vesicular Acetylcholine Transporter in Cholinergic Nerve Terminals and Its Targeting to a Specific Population of Small Synaptic Vesicles.” *Proceedings of the National Academy of Sciences* 93(8): 3547–52.

Zhang, Wenjun et al. 2017. “Fully Automated 5-Plex Fluorescent Immunohistochemistry with Tyramide Signal Amplification and Same Species Antibodies.” *Laboratory Investigation* 97(7): 873–85.