Enhanced Cholinergic Interneuron Striatal Density Demonstrated in a SAPAP3 Knockout: An Indirect Quantification of Elevated Acetylcholine Levels in an OCD Mouse Model

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Introduction

OCD stands for Obsessive Compulsive Disorder. It's characterized by obsessive thoughts leading to compulsive action. Although not detrimental to a person's health, it severely inhibits their quality of life. About 2.3% of the population is diagnosed with OCD. However, there are limited treatments due to our incomplete understanding of its pathophysiology and circuitry. The cortico-striato-thalamo-cortical loop has been implicated in the disease as it relates to reward learning. The striatum is a key mediator of compulsive behaviors and is a prime region of interest when studying OCD as a result. Within the striatum, 95% of cells are Spiny Projection Neurons (SPNs), which are composed of 2 distinct types: direct spiny projection neurons (dSPNs) which promote action, and indirect spiny projection neurons (iSPNs) which inhibit action. Preliminary data from our lab suggest that dSPNs are preferentially more excited in OCD mouse models than dSPNs in WT mice, and iSPNs are preferentially less excitable in OCD mouse models than in WT mice. Excitability is the ability of these cells to fire an action potential, so if dSPNs are more excited and iSPNs are less excited, the "go pathway" is induced resulting in compulsive behaviors in OCD patients.

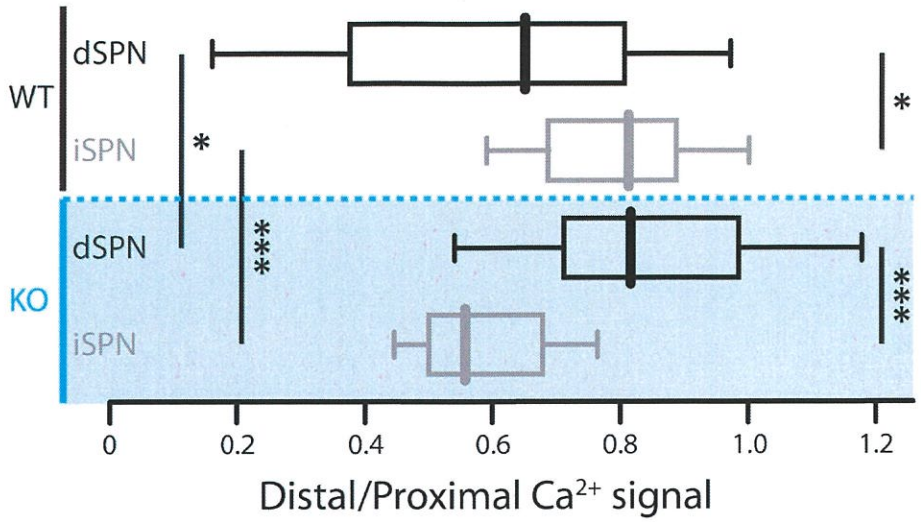

Figure 1: SAPAP3 KO mice's dSPNs and iSPNs excitability are compared to WT's dSPNs and iSPNs. The distance backpropagating action potentials reached in each cell was measured and analyzed, and it reveals that dSPNs are more excitable, and iSPNs are less excitable in OCD modeled mice. Source: Preliminary data

The neurotransmitter acetylcholine (ACh) is found heavily in the striatum and is thought to be implicated in OCD. Cholinergic Interneurons (ChIs) compromise only 1-2% of striatal cells, but they are the principal source of striatal ACh. One mechanism of how SPNs have an altered excitability is through ACh acting as a modulator. iSPNs have M1 (muscarinic) receptors that promote excitability when ACh binds, and dSPNs have M4 receptors that decrease excitability when ACh binds.

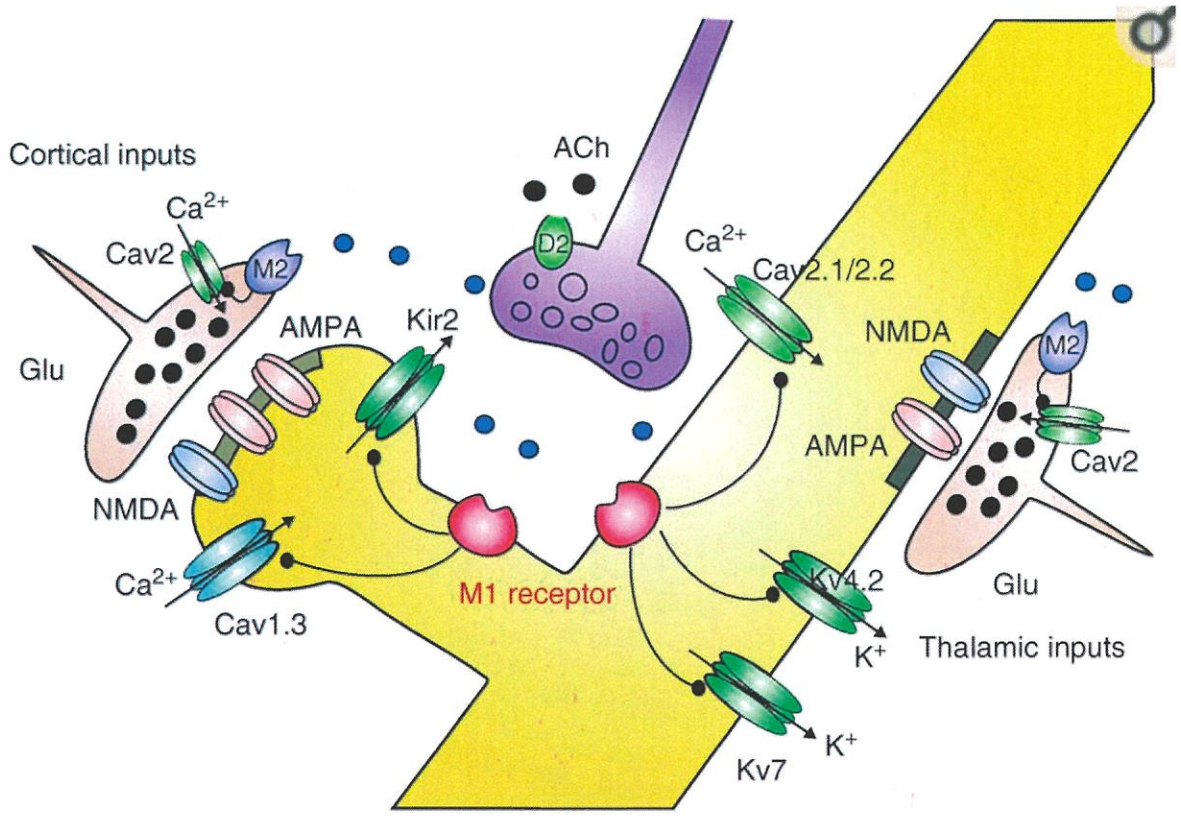

Figure 2: When ACh binds to M1 receptors on iSPNs, calcium ion channels open up allowing calcium ions to flow into the cell, and potassium ion channels are opened, allowing potassium ions to flow out of the cell and further depolarize the cell. Both these actions increase the spiking activity of iSPNs / make it more excitable. Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3138897/#R32

Any change in ACh levels has been correlated with a rise in OCD-like symptoms, and the researchers altered these levels by directly manipulating ChIs - however - no one has observed if ChIs themselves directly participate in the OCD model. Since we've observed that iSPNs are less excitable and that more ACh should make them more excitable due to binding to M1 receptors, we did immunohistochemistry on WT versus KO mouse brains to test indirectly for altered ACh levels by directly measuring ChIs. We also stained for ChIs directly to gather more evidence on if ChIs themselves are directly implicated in OCD's circuitry.

Materials and Methods

Rationale of OCD Mouse Models

SAPAP3 deficient mice were used as OCD mouse models. Previous studies have demonstrated altered cortico-striato-thalamo-cortico functioning levels within SAPAP3 KO mice, which is characteristic of movement disorders like Parkinson's as well as mental disorders dealing with the dopaminergic reward pathway, such as Attention Deficit Hyperactivity Disorder (ADHD), and OCD patients. SAPAP3 is a scaffolding protein on the postsynaptic ends of excitatory glutamatergic synapses on SPNs. Previous literature observes that SAPAP3 KO mice exhibit an increase in anxiety-like behaviors, such as an increase in grooming, leading to a visible increase in lesions, more time spent on the edges of their room rather than in the center, and less time in a light chamber. Corticostriatal synaptic defects were also observed as SAPAP3 mutants had lower field excitatory postsynaptic potentials (fEPSPs) and altered NMDA subunits which change signalling cascades and thus how neurons within this circuit to respond. Altered corticostriatal defects and repetitive actions demonstrating anxiety like behaviors are characteristic of OCD, lending SAPAP3 KO mice to be used as an OCD model.

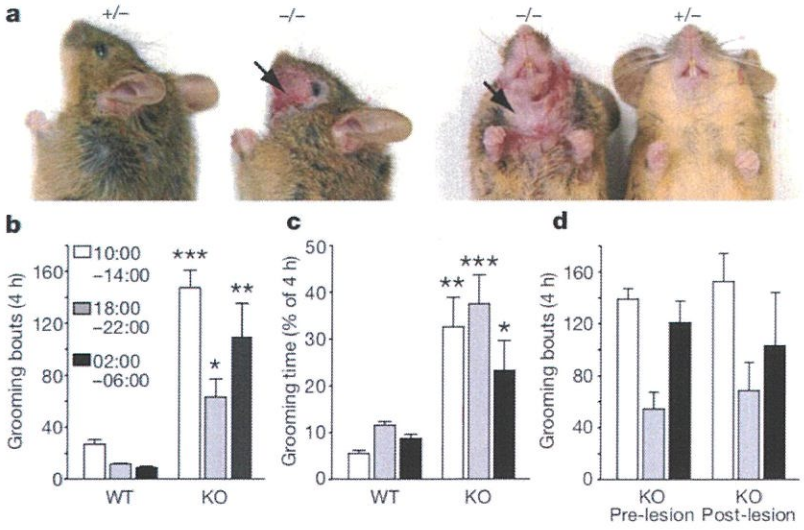

Figure 3: SAPAP3 knockout mice exhibit increased grooming bouts which is representative of compulsive behaviors that is found in OCD patients. Visual self-inflicted lesions from grooming compared to wild-type mice are pictured (a). Increased grooming time was also observed in knockout mice (b,c,d). Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2442572/

Immunohistochemistry

In order to determine if ChIs directly participate in OCD's circuitry, we performed immunohistochemistry on 16 mice brains, staining for choline acetyltransferase (ChAT) in order to find ChIs because ChIs are responsible for the striatum's ACh, and ChAT is the enzyme that synthesizes ACh, so it is highly found in ChIs. We also performed immunohistochemistry to indirectly quantify ACh levels to gather support or deterrence about the hypothesis that ACh binding is modulating the excitability of SPNs. An increase in ChIs is an increase in ACh measured through ChIs and not directly ACh, thus rendering it an indirect qualitative measurement, given we cannot quantify the actual amount of ACh within a given ChI, we can only determine if a neuron is a ChI or not through this experimentation. We were blinded to the genotypes of the mice in order to limit bias during the analysis of our products. Each brain was

cryocut in 20μ intervals coronally and chilled in the fridge at -4℃ until their use. The protocol for each brain's staining is:

1. PBS rinse (10 min RT)
2. Block in 3% NDS, .1% triton (2hrs RT) (1.5mL/well)
3. 1:1000 Gt α ChAT in .5% NDS, .1% triton (overnight 4℃)
4. Wash in .5% NDS, .1%triton, (3 x 10min RT)
5. 1:2000 Dk α Gt in .5% NDS, .1% triton (2-3hr RT)
6. Wash in .5% NDS, .1% triton (3 x 10min RT)
7. PBS rinse (3+ min RT)
8. Mount 8 slices/well

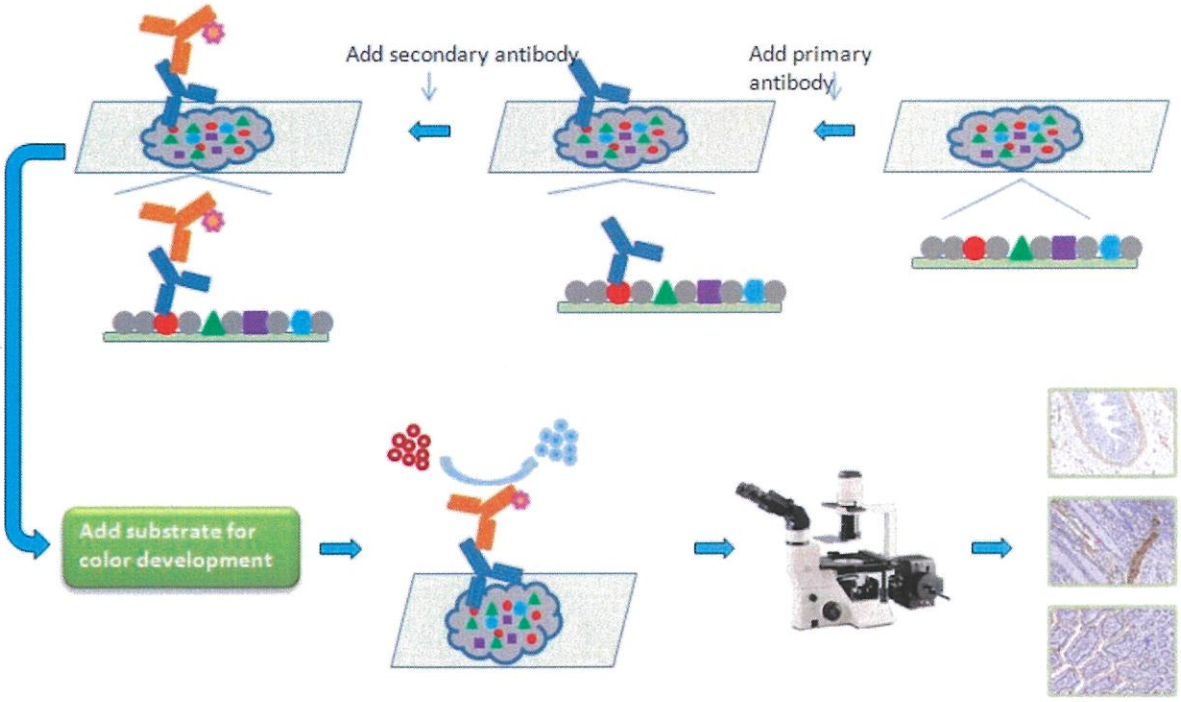

Figure 4: Immunohistochemistry works via 2 antibodies binding to specific targets. The primary antibody binds directly the choline acetyltransferase by binding directly to the enzyme's cell receptors. The secondary antibody then binds to the first, and the second antibody possesses a fluorophore, which is responsible for the emittance of light and therefore observable stain. Source :https://industrydailyobserver.com/2019/03/14/new-research-immunohistochemistry-ihc-market-analysis-top-companies-new-technology-demand-and-opportunity-analysis-by-2024/71430/

Analysis

Each slice was photographed under a microscope in 31 z-stacks because cells are 3-D and thin, so there may be cells under the superficial layer because each slice is 20 microns thick. After being uploaded to the computer, ImageJ was used for analysis. The cholinergic interneuron channel was made red, and the mu-opioid channel was made green -- both of these arbitrary colors. The striatum was outlined via the anterior commissure and lateral ventricles as landmarks.

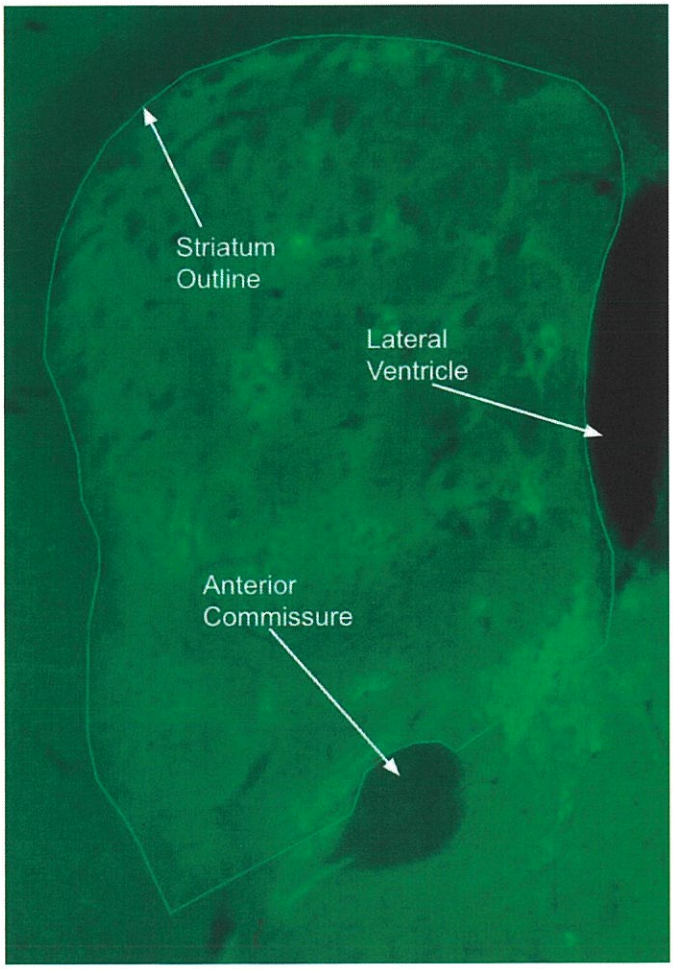

Figure 5: Each slice of the mice brains had its striatum outlined. The anterior commissure and lateral ventricle were used as landmarks for the outlining process. Source: Student generated image

After the striatum was outlined, ChIs were analyzed through the thresholding of them in the ChAT red channel for circularity and light intensity. After, they were manually checked to make sure each ROI was in fact a ChI. Each channel was then merged into one composite image, and all striatum area and ChI count was put into graphs.

Results

When KO slices were tested separately compared to the corresponding bregma value for WT mice, no statistical significance was found for ChI differences, or striatal density differences.

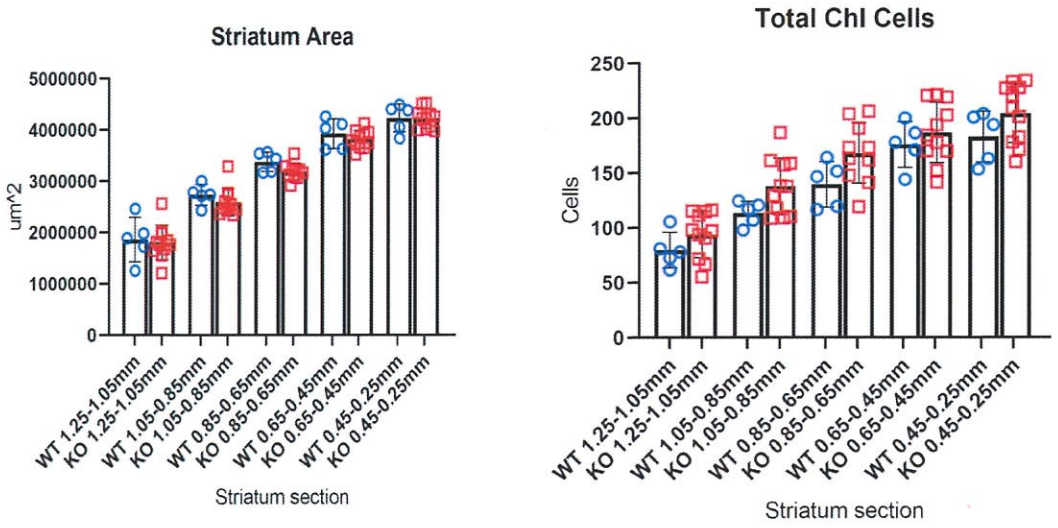

Figure 6: Striatum area and ChI cell count were not statistically significant when Mann-Whitney ($p < .05$) t-tests were performed for each slice's corresponding bregma value. Source: Student generated image

Consequently, the ChI striatal density difference was not statistically significant when each slice was directly compared to the concurrent bregma value of the other experimental group.

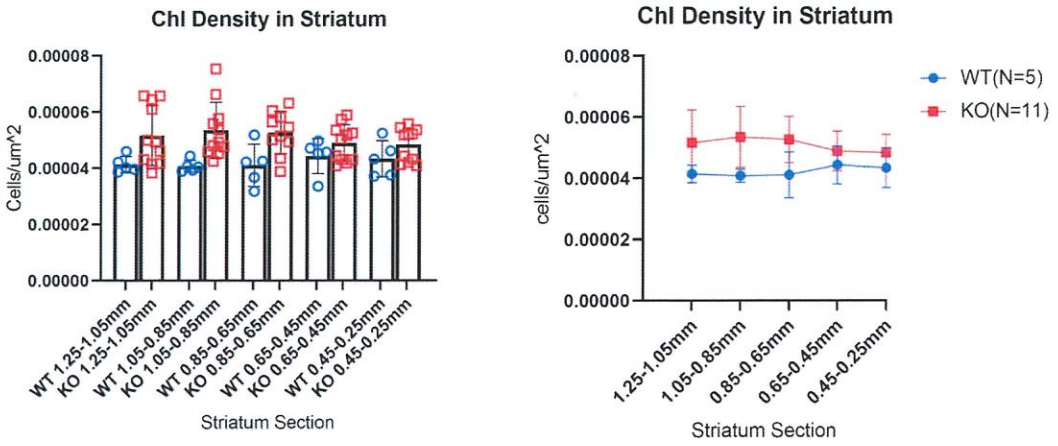

Figure 7: No statistical significance was observed when directly comparing ChI density differences between corresponding bregma valued slices in KO vs WT mice. Source: student generated image.

However, when the total average of ChI density for KO was compared to the total average ChI density for WT mice statistical significance was observed (2-tailed unequal variance Mann-Whitney T-test; $p < .05$).

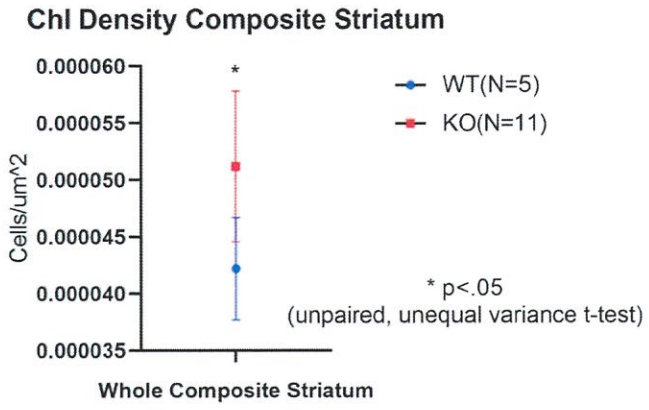

Figure 8: When the ChI density of the whole striatum for KO mice was compared to that of WT mice, statistical significance was observed. Source: Student generated image

Discussion

Our data demonstrates that there is a higher ChI density in OCD mouse models when compared to WT mice. Consequently, we gathered direct evidence that ChIs are implicated in OCD's circuitry (form glutamatergic synapses with SPNs) because there is a statistical difference between their densities, and the only difference between the mice are their genotypes (KO vs WT). However, there is still a possibility that these changes are the result of circuit-wide adaptations due to the loss of the SAPAP3 gene, so we cannot definitevly say that ChIs are directly involved in OCD's etiology because the findings we observe may be a reactionary effect as a result of circuit changes/adaptations. Our data also indirectly demonstrates a decrease in ACh levels within the striatum for OCD mice when compared to WT mice. Since ChIs are the principal secretor of ACh in the striatum and we found an increased density of them in KO mice, we indirectly quantified an increase in ACh levels. However, ACh was not directly observed, so we can't definitely say that ACh is increased in OCD mice, but we can make the argument more credible. Therefore, the hypothesis that SPN excitation modulation was a result of ACh is less likely, although not impossible. We did not gather direct evidence for decreasing ACh, and the proposed excitation modulation from ACh is a result of ACh binding to SPNs, which can be decreased with a rise of ACh in the presence of nAChR antagonists - and this is not tested for either. Thus, the hypothesis is still plausible, but it is a lot less likely.

Conclusion

Collectively, our results shed light on future directions to study OCD: 1) Gather direct evidence through electron microscopy/ 2-photon microscopy to observe if ChIs form glutamatergic synapses with SPNs, and if they do how it affects them. 2) Rather than spend time and money on treating ACh as the direct role for SPN modulated excitability, test other possible mechanisms for changing SPN excitability (ex: dopamine) that may indirectly incorporate the indirect increased ACh finding.

Future Work

Future research must be done to be able to concretely reject and/or accept the hypothesis that ChIs are implicated in OCD's circuitry and ACh is responsible for the changing SPN excitability. For further validation of our results, immunohistochemistry should be re-done using DAPI for total automatization of ChI cell count to significantly reduce any statistical error. Striosomes should also be measured and see if ChIs preferentially cluster inside, around, or outside of them because cells within striosomes project to dopaminergic neurons in the substantia nigra pars compacta which goes back to the striatum, compared to matrix projections to the thalamus. ChIs should be tested for expression of the SAPAP3 gene to be certain it is implicated in OCD's circuits directly. Our results shed light on a more likely explanation for SPN excitation: a rise in ACh leads to more binding on dopamine axon fibers in the substantia nigra pars compacta which in turn release more dopamine into the striatum which then binds to D1 receptors on iSPNs which decreases their excitation and D2 receptors on dSPNs which increases their excitation.

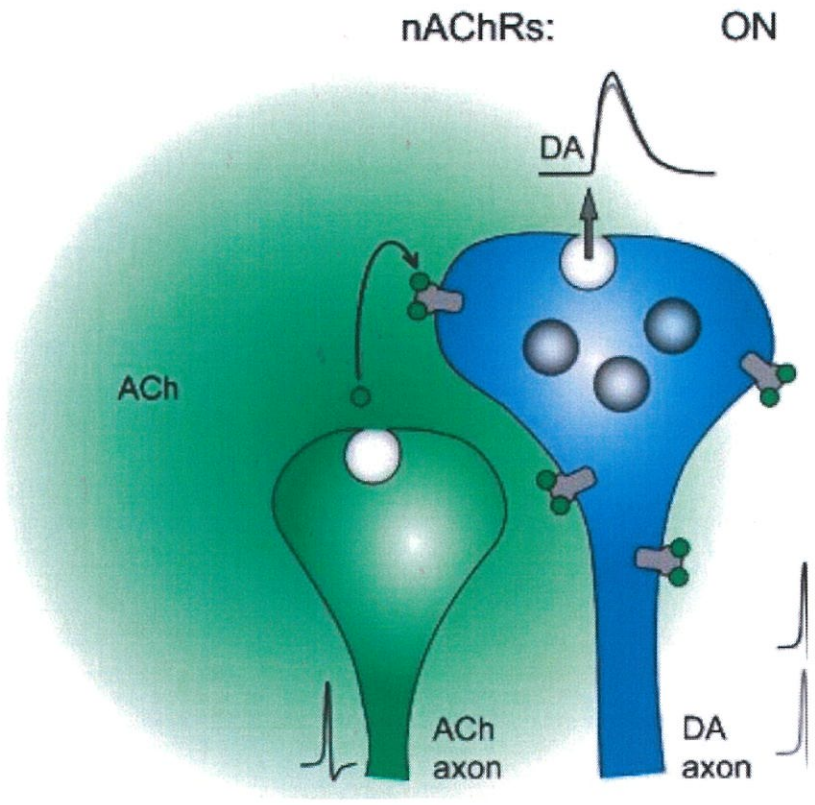

Figure 9: ACh binding to dopamine axon nicotinic receptors leads to the release of more dopamine into the striatum, which then binds to D1 and D2 receptors on iSPNs and dSPNs respectively. Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2268048/

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