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Reporting the latest news in Molecular Surgery

Targeted lesioning reveals role of nucleus incertus in the anxiogenic effect of buspirone

By Jigna Rajesh Kumar, [a, b, c, d] *Ramamoorthy Rajkumar,* [a, b, c] *Liying Corinne Lee,* [a, b, c] *Gavin S. Dawe* [a, b, c, d]

[a] *Dept Pharmacology, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, 117600, Singapore*

[b] *Neurobiology and Ageing Programme, Life Sciences Institute, National University of Singapore, 117456, Singapore*

[c] *Singapore Institute for Neurotechnology (SINAPSE), 117456, Singapore*

[d] *NUS Graduate School for Integrative Sciences and Engineering (NGS), National University of Singapore, 117456, Singapore*

The nucleus incertus (NI), located strategically at the prepontine brainstem, has widespread connections across the forebrain to various structures involved in arousal, behavioral regulation, anxiety, appetite, and cognition.[5, 6, 9, 11-12, 18-20] The NI expresses one of the highest density of corticotropin releasing factor receptor 1 (CRF1) in the brain. which raised interest in this structure and suggested its possible role in the extra-pituitary behavioral stress response.[16] The NI is the chief source of the neuropeptide relaxin-3, and the NI/relaxin-3 system is highly conserved phylogenetically, pointing to a critical functional role that is presently not well understood.[15, 22]

CRF-saporin (CRF-SAP; Cat. #IT-13) was stereotaxically injected into the NI to enable selective lesioning of the CRF1-expressing NI cells (Fig. 2, Page 6).[13] This procedure established at our laboratory was found to show significant reduction in the expression of CRF1, relaxin-3, GAD 65 as well as relaxin-3 in a representative target structure, the medial septum.[13] Based on the anxiogenic effect of CRF-SAP lesioning of the NI, as depicted by the significantly reduced time spent, and entries into the open arms of the elevated plus maze, it can be inferred that the NI may act to reduce anxiety physiologically (Fig. 1).

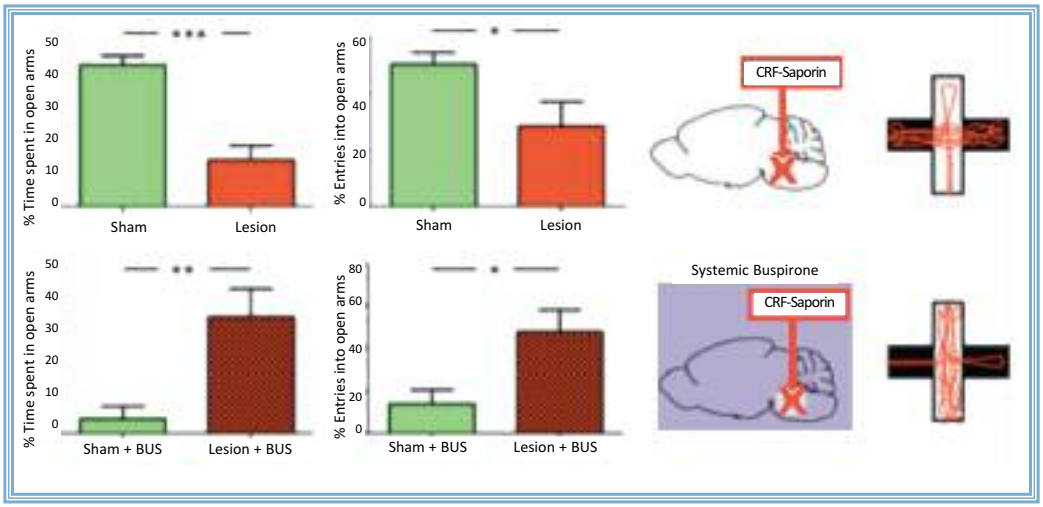


Fig. 1. Rats stereotaxically injected with CRF-SAP (Lesion) or Saporin (Cat. #PR-01; Sham) in the NI are tested in the elevated plus maze. Lesioning of the NI has an anxiogenic effect based on the reduced time spent and number of entries into the open arms. Systemic buspirone (3mg/kg; BUS) treatment reduces the anxiety levels of lesioned rats, increasing the time spent in, and number of entries into, the open arms.

(continued on page 6)

Strategic Partners

Denise Higgins - President

This issue of Targeting Trends brings an exciting report of new partners that will bring more products, more expertise, and more service to our customers around the world. The primary purpose for making this change is to better facilitate the various functions that serve you, our customer. This restructure will also challenge and reward our long-time employees with the responsibility of managing their own strategic unit.

Four partners with unique strengths combine to complete the Targeting Puzzle:

- Advanced Targeting Systems will continue to provide excellent Customer Service
- BioSyntheSys will provide quality conjugation services
- CytoLogistics will provide consistent, reliable antibody production, flow cytometry, and other laboratory services
- TLC Shipping & Storage will ensure all products are stored, packaged and shipped properly.

Brian Russell, celebrating his 16th anniversary at ATS on October 2nd, will be managing **BioSyntheSys**. His team will provide custom conjugation services: ADCs, saporin conjugates, biotinylations, fluorescent labeling, etc. Log on to ***BioSyntheSys.com*** to enter your contact information to discuss your next conjugation service.

Leonardo Ancheta, celebrated his 13th anniversary at ATS on September 2nd, and will be managing **CytoLogistics**' contract services division. Leonardo's team will provide flow cytometry services, cell culture, laboratory assays, antibody production, and GLP contract services. Log on to ***CytoLogistics.com*** to reserve your next service.

Doug Lappi (Founder and President Emeritus of ATS) will be heading up the Research Division of **CytoLogistics**. His team will work on product development and offer stellar laboratory expertise and consulting in Biochemistry, Molecular Biology, assay development, Cell biology and standard laboratory skills.

Tom Cobb and Chelsea Friedman will be co-managing **TLC Shipping & Storage**. Tom has extensive experience with shipping logistics and will ensure that all orders are packaged, shipped and tracked to their destinations worldwide. Chelsea is a scientist with an excellent background in storage and handling of research reagents (biologics, antibodies, etc.) and will manage the inventory tracking of multiple temperature storage units. Log on to ***TLCshipstorage.com*** to see the services they have to offer.

As for me, I will be continuing to manage **Advanced Targeting Systems** with an emphasis on administrative support for all of our partners. By consolidating all the administrative functions (customer orders, sales & marketing, purchasing, payroll, licensing, legal, etc.) for multiple entities, there is greater efficiency and organization in the processes. We are all excited about the prospects for each of our partners and for the greater service we can provide to our customers.

TLC Shipping & Storage is a San Diego-based service company experienced in logistics of managing research-grade laboratory supplies. Advanced Targeting Systems has partnered with TLC to provide:

- Inventory tracking
- Storage
- Packaging
- Shipping worldwide

TLC Shipping specializes in transportation and logistics for perishable commodities. TLC ships chilled, frozen, and shelf-stable goods to destinations around the world. Customers can rely on TLC to handle every aspect of packaging and shipping research reagents. TLC communicates each step of the process with visibility to track and trace every shipment.

TLC Storage provides short and long term temperature-controlled storage for non-hazardous research samples, production batches and products. Experienced associates manage your inventory from receipt through distribution TLC provides customers with peace of mind that inventory is well-protected, organized and secure through regular audited inventory reports and updates.

ATS Toolbox - New Partners

CytoLogistics is the Contract and Research Services partner to Advanced Targeting Systems. This new CRO (Contract Research Oorganization) used the foundation laid out by Cytometry Research LLC (established in 1993 to offer flow cytometry services to San Diego customers), and has expanded to help fulfill more encompassing Life Science and Preclinical Research needs.

CytoLogistics has a scientific staff with expertise to provide a wide range of services:

- Flow Cytometry (Design, Analysis, Sorting)
- Immunocytochemistry, immunohistochemistry
- *In vitro* laboratory services (cell culture, cell staining/preparation)
- *In vitro* cell–based assays (cytotoxicity, proliferation, compound/drug screening)
- Cell analysis and protein characterization (SDS-PAGE, Immunoblotting, ELISA, BCA, HPLC).
- Basic research (biochemistry, molecular biology)

Preclinical research often requires a higher level of service than other programs. To meet this need, **CytoLogistics** has GLP processes in place to ensure consistently reliable results, a quality assurance program to provide independent confirmation of procedures and results, and full documentation samples, data, and testing results provided with each service.

So, whether your laboratory needs extra hands for cell culture and basic research or you need professionally-designed and executed cell services, CytoLogistics delivers encompassing service and fluidity.

BioSyntheSys (BSS) is the preferred contracted partner of Advanced Targeting Systems for all crosslinking needs, including their well-established line of targeted Saporin conjugates. It is the mission of BioSyntheSys to provide academic and industry R&D clients with access to custom-crosslinking of proteins, peptides, and activated small molecules via boutique contract services. Their team of specialists leverage an extensive knowledge-base and catalog of strategies to make a complex process simple, allowing BSS clients to stay focused on what they do best.

- Antibody-drug conjugates
- Saporin and saporin-related conjugates
- Biotinylations
- Fluorescent labeling
- Linker design and consultation

If your research or drug development program needs a production partner or perhaps a company with specialized skills to outsource projects to, contact BioSyntheSys to discuss your next big discovery.

Recent Publications & References

Reviewed by Chelsea Friedman

Neural activity promotes long-distance, target-specific regeneration of adult retinal axons.

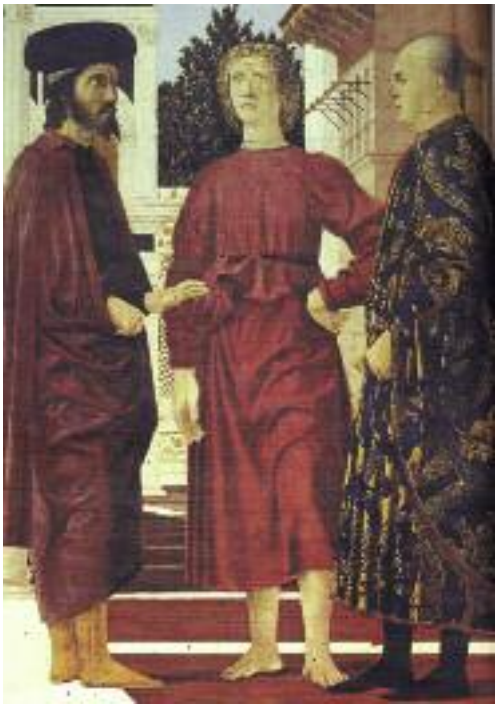
Lim JH, Stafford BK, Nguyen PL, Lien BV, Wang C, Zukor K, He Z, Huberman AD.
Nat Neurosci 19(8):1073-1084, 2016.

Axons in the CNS fail to regenerate after injury. Scientists sought to identify strategies that would allow retinal ganglion cell (RGC) axons to regenerate in the eye-to-brain pathway, and if that was possible, whether the axons could reconnect with their correct targets and restore visual function. It was previously shown that increasing mTOR signaling could trigger RGC axon regeneration. Several conditions were tested, but combining increased mTOR signaling and then exposing mice to high-contrast visual stimulation daily for 3 weeks after optic nerve crush resulted in long distance RGC axon regeneration, re-innervation of the brain, and partial recovery of a subset of visual behaviors. A 1:1000 dilution of Anti-Melanopsin (Cat. #AB-N38) was used for the immunohistochemical analysis of retinas, optic nerves, and brain tissue.

Effective antitumor therapy based on a novel antibody-drug conjugate targeting the Tn carbohydrate antigen.

Sedlik C, Heitzmann A, Viel S, Ait Sarkouh R, Batisse C, Schmidt F, De La Rochere P, Amzallag N, Osinaga E, Oppezzo P, Pritsch O, Sastre-Garau X, Hubert P, Amigorena S, Piaggio E.
Oncoimmunology 5(7):e1171434, 2016.

Scientists wanted to study the potential of Chi-Tn, a monoclonal antibody against a glycol-peptidic tumor-associated antigen, as an anticancer antibody-drug conjugate. They demonstrated that Chi-Tn specifically targeted tumor cells *in vivo*, using flow cytometry and deconvolution microscopy to show that Chi-Tn is rapidly internalized. Chi-Tn-SAP (ATS Custom Services) effectively killed Tn-positive cells, but had no effect on Tn-negative cells. Saporin (Cat. #PR-01) was used as a control. The cytotoxicity of the Chi-Tn-SAP correlated with the level of tumoral Tn expression.



Limiting glucocorticoid secretion increases the anorexigenic property of Exendin-4.

Lee SJ, Diener K, Kaufman S, Krieger JP, Pettersen KG, Jejelava N, Arnold M, Watts AG, Langhans W.
Mol Metab 5(7):552-565, 2016.

Glucagon-like peptide-1 (GLP-1) analogs lower blood surgar levels and cause a loss of appetite. Exendin-4 (Ex-4) is a GLP-1 receptor agonist, and also increases glucocorticoid secretion. Several tests were conducted to determine if the released glucocorticoids interact with Ex-4's anorexigenic effect. One method involved ablating hindbrain catecholaminergic neurons by stereotaxically injecting 42 ng of Anti-DBH-SAP (Cat. #IT-03) bilaterally into the paraventricular nucleus of the hypothalamus in rats. Animals were injected with equimolar concentrations of unconjugated Saporin (Cat. #PR-01) as a control. Anti-DBH-SAP lesions reduced the efficacy of Ex-4 to increase corticosterone secretion but increased the anorexigenic effect, indicating that Ex-4-dependent corticosterone secretion opposes Ex-4's actions. Anti-DBH-SAP lesions increased Ex-4's ability to reduce food intake and body weight.

Brain penetration, target engagement, and disposition of the blood-brain barrier-crossing bispecific antibody antagonist of metabotropic glutamate receptor type 1.

Webster CI, Caram-Salas N, Haqqani AS, Thom G, Brown L, Rennie K, Yogi A, Costain W, Brunette E, Stanimirovic DB.
FASEB J 30(5):1927-1940, 2016.

To generate a BBB-transmigrating antibody that could be reformatted to full IgG, scientists started with the BBB-crossing llama single domain antibody FC5. Standard phage display protocols were used to isolate single-chain variable fragments (scFv) from the FC5-scFv library. 6His Mouse Monoclonal antibody (Cat. #AB-213) was used to assess cell binding of scFvs of FC5 using fluorescence microvolume assay technology. An scFv that competed with FC5 binding was selected for further testing. An antibody antagonist of the metabotropic glutamate receptor-1 was fused with this scFv antibody fragment (BBB-mGluR1) and tested in an *in vitro* BBB model. The resulting bispecific antibody retained selective mGluR1 binding and saw a 20-fold enhanced rate of transcytosis across the BBB compared to fusion with control antibody fragment. Intravenous injection of BBB-mGluR1 had analgesic properties in a rat model of persistent inflammatory pain.

Lysophosphatidylcholine acyltransferase 1 protects against cytotoxicity induced by polyunsaturated fatty acids.

Akagi S, Kono N, Ariyama H, Shindou H, Shimizu T, Arai H.
FASEB J 30(5):2027-2039, 2016.

Dietary consumption of polyunsaturated fatty acids can influence the degree of fatty acid unsaturation in membrane phospholipids, and consequently membrane-associated functions. Scientists set out to investigate how mammalian cells change their membrane lipid composition in response to loading with excess polyunsaturated fatty acids (PUFAs). Lipidomic analysis showed that PUFA treatment induces production of dipalmitoylphosphatidylcholine (DPPC). By

(continued on page 5)

Recent Publications & References

(continued from page 4)

suppressing phospholipid metabolism-related genes by RNA interference, they found that Lysophosphatidylcholine acyltransferase 1 (LPCAT1) was involved in DPPC production. To reveal the role of DPPC produced by PUFA treatment, HeLa cells were transfected with an siRNA against LPCAT1 to reduce its protein expression. The cells were lysed after treatment with a PUFA and subjected to western blot analysis using a 1:1000 dilution of Anti-SCD-1 (Cat. #AB-259) as the primary. SCD-1 desaturates the substrate of LPCAT1 for producing DPPC. PUFAs significantly reduced both the protein and mRNA expression of SCD-1. They showed that inhibiting DPPC production by LPCAT1 knockdown enhanced apoptosis, suggesting that DPPC produced via LPCAT1 protects against PUFA-induced cytotoxicity.

Selective noradrenaline depletion impairs working memory and hippocampal neurogenesis.

Coradazzi M, Gulino R, Fieramosca F, Falzacappa LV, Riggi M, Leanza G.

Neurobiol Aging 48:93-102, 2016.

Neuronal loss in the locus coeruleus (LC) of Alzheimer's patients is well known, but the contribution of LC-derived noradrenergic afferents to learning and memory function is unknown. To model noradrenergic neuron degeneration in the LC, rats were bilaterally injected directly into the LC with 0.2 mcg of Anti-DBH-SAP (Cat. #IT-03). Lesioned and sham-lesioned animals were tested behaviorally and exhibited robust working memory deficits, yet lesioning did not affect reference memory. They concluded that ascending noradrenergic afferents might be involved in more complex aspects of working memory, possibly via newly generated progenitors in the hippocampus.

Nucleus incertus contributes to an anxiogenic effect of buspirone in rats: Involvement of 5-HT1A receptors.

Kumar JR, Rajkumar R, Lee LC, Dawe GS.

Neuropharmacology 110(Pt A):1-14, 2016.

To see if the NI is necessary for the anxiogenic effects of high doses of buspirone, rats were bilaterally injected with 86 ng of CRF-SAP (Cat. #IT-13) into the NI. Blank-SAP (Cat. #IT-21) was used as a control. (See Cover Article)

Substituting mouse transcription factor Pou4f2 with a sea urchin orthologue restores retinal ganglion cell development.

Mao CA, Agca C, Mocko-Strand JA, Wang J, Ullrich-Luter E, Pan P, Wang SW, Arnone MI, Frishman LJ, Klein WH.

Proc Biol Sci 283(1826):20152978, 2016.

The sea urchin genome contains SpPou4f1/2, a distant orthologue of Pou4f2, but they have no obvious eyes and their photoreceptors are located around their tube feet disc. Scientists replaced genomic Pou4f2 with an SpPou4f1/2 cDNA to see if SpPou4f1/2 could support RGC development in mice. Mice expressing SpPou4f1/2 developed retinas that looked like wild-type mice. Immunolabeling of retinas with a 1:1000 dilution of Anti-Melanopsin (Cat. #AB-N39) showed the presence of many well-bundled axons emanating from SpPou4f1/2-expressing RGCs. Electroretinogram recordings from these mice indicate that their RGCs are functionally active. These results suggest that there is a high degree of functional conservation between the two genes.

GABA-A receptor activity in the noradrenergic locus coeruleus drives trigeminal neuropathic pain in the rat; contribution of NAalpha1 receptors in the medial prefrontal cortex.

Kaushal R, Taylor BK, Jamal AB, Zhang L, Ma F, Donahue R, Westlund KN.

Neuroscience 334:148-159, 2016.

The goal of this study was to investigate the role of the locus coeruleus (LC) in a rat orofacial pain model of trigeminal neuropathy induced by chronic constrictive injury of the infraorbital nerve (CCI-ION). Noradrenergic (NA) neurons were lesioned with 5-mcg injections of Anti-DBH-SAP (Cat. #IT-03) into the left lateral ventricle. Mouse-IgG-SAP (Cat. #IT-18) was used as a control. After ablation of NA neurons there was a notable increase in the mechanical threshold of Von Frey filaments tested on whisker pads compared to control animals. Injecting a GABAA receptor antagonist into the LC after injury had an inhibitory effect on nerve injury induced hypersensitivity. They concluded that GABAA-mediated activation of NA neurons during CCI-ION can facilitate hypersensitivity through NAα1 receptors in the mPFC, and that the LC is a chronic pain generator.

A2 noradrenergic neurons regulate forced swim test immobility.

Nam H, Kerman IA.

Physiol Behav 165:339-349, 2016.

Researchers discovered relative hyperactivation in the locus coeruleus of Wistar-Kyoto (WKY) depression model rats compared to the genetically related Wistar rats when exposed to one- and two-day forced swim tests (FSTs). A2 noradrenergic neurons of Winstar rats were lesioned by injecting 2.2 mcg of Anti-DBH-SAP (Cat. #IT-03) into the nucleus tractus solitaris (NTS). Lesioned rats exhibited increased FST immobility on both days of the test, similar to natural WKY behavior in the same test.

Saponins from Saponaria officinalis L. Augment the Efficacy of a Rituximab-Immunotoxin.

Gilabert-Oriol R, Thakur M, Haussmann K, Niesler N, Bhargava C, Gorick C, Fuchs H, Weng A.

Planta Med 2016.

Investigators wanted to know if triterpenoidal saponins that come from *Saponaria officinalis* could increase the therapeutic affect of Rituximab-Saporin. In the presence of saponins, Rituximab-Saporin had a 700-fold increase in efficacy. Concentrations of 0.0001-1nM of Anti-CD22-SAP (Cat. #IT-37) and 0.001-10nM of Anti-CD25-SAP (Cat. #IT-24) were also tested *in vitro* with saponins for comparison. They saw a 170-fold and 25-fold increase in cytotoxicity, respectively.

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Targeted lesioning reveals role of nucleus incertus in the anxiogenic effect of buspirone

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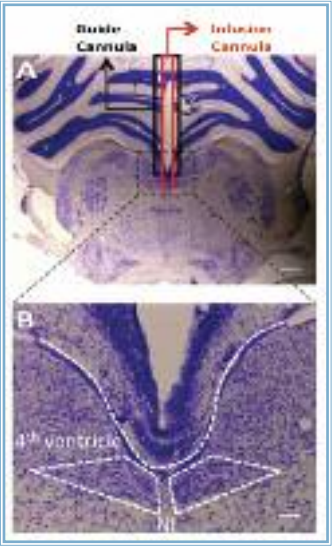

Fig. 2. Representative images showing cannula position in the NI at 2X (A; scale=1 mm) and 10X (B; scale = 200 μm).

We further utilized this technique to determine if the NI was involved in mediating the anxiety-modulating effects of buspirone, a clinically prescribed novel anxiolytic whose mechanism of action is not well understood.[3-4] Buspirone is a anxioselective drug that acts specifically on symptoms of anxiety without affecting cognition, motor ability, and reward pathways thus indicating that it likely acts on structures that regulate physiological anxiety. Buspirone is a 5-HT1A partial agonist and a D2 receptor antagonist;[(14)] both receptors are expressed in the NI.[13, 17] Buspirone tends to show anxiolytic effects at a narrow low dose range and anxiogenic effects at a wide high dose range, the latter effect being robust and reproducible.[1, 4, 7, 10, 21] The anxiolytic effects are widely thought to be mediated by the agonism of the 5-HT1A autoreceptors at the raphe nuclei, particularly the median raphe.[2, 8, 23] A 3 mg/kg intraperitoneal dose of buspirone was found to induce a strong anxiogenic effect on various anxiety paradigms, namely the elevated plus maze, the open field, and the light-dark box.[12] This dose was also found to robustly induce c-Fos expression and therefore activate the NI. The anxiogenic effect of systemic buspirone was attenuated when the NI was lesioned by CRF-SAP, thus indicating that the NI plays a role in the effects of buspirone (Fig. 1). Infusing buspirone (5 mcg) into the NI produced increased anxiety as well, suggesting that buspirone may be acting directly on the NI.[12] Pharmacological interaction studies conducted with a 5-HT1A antagonist, NAD 299, and D2/D3 agonist, quinpirole, indicated that these effects are mediated through the 5-HT1A receptors. Intra-NI infusion of NAD 299 attenuated the anxiogenic effects of systemic buspirone while intra-NI quinpirole did not have any effect.[12] Therefore, the NI is likely to be part of the physiological anxiety circuit and the 5-HT1A receptors may be particularly important in mediating this function.

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Talking about Targeting

New Additions

In this quarter's Targeting Talk, ATS is proud to talk about more than new products, a new Product Manager! Her name is Alena Bishop and although originally from the San Francisco Bay Area, she recently graduated from UCSD in June of this year. With a newly minted degree in Molecular Biology and ATS's need for a Research Associate with just such a pedigree, it has proved to be an excellent match. Since starting in July, Alena has taken quickly to the innovative work being done in the lab and appreciates participating in the development of products that play such an important role in the everyday research of so many scientists globally. Eager to dive into the customer-facing aspects of our products, she looks forward to engaging with each of you regarding any technical questions you may have.

She's here to tell you, in her own words, what she is working on and what products to look forward to:

"One of the projects I have been working on involves a novel use of specific strains of Clostridium botulinum *that inhibits cells by blocking the release of neurotransmitters. This effect is long-lasting but not permanent, and this attribute gives the toxin great potential as a new targeted payload. This toxic payload could be used to temporarily prevent cell function without actually killing the cells, and coupled with ATS's targeting technology this treatment could be administered specifically to cells of interest. Avoiding killing the cells completely would provide a life-like model to study the effects of temporary loss of cell function in organisms. After a certain period of time, the effects of the toxin would disappear, restoring normal neuronal cell function. My immediate goal is to put the finishing touches on an assay kit for quantifying the activity of Clostridium botulinum useful for a variety of strains. Look for this activity assay kit to be released in the coming quarter!"*

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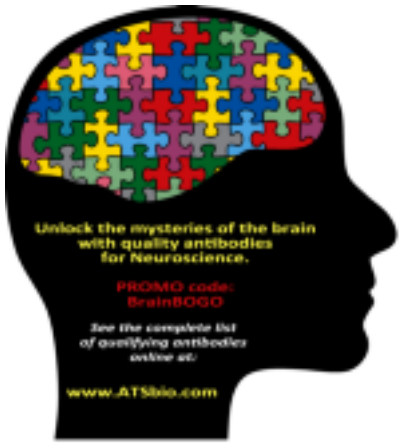

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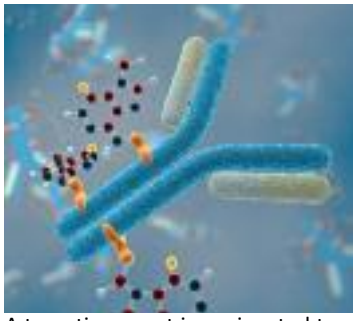
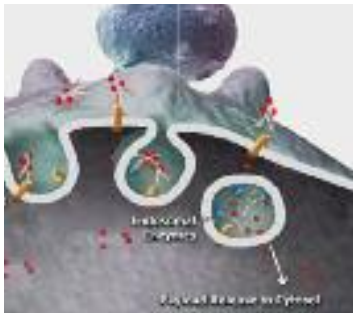


A targeting agent is conjugated to a payload. Here the conjugate is an antibody linked to a small molecule.

The targeted conjugate binds only the cells that are specific to the antibody causing the receptor to internalize.



The internalized conjugate is digested by enzymes releasing the payload into the cytosol. The payload is then free to alter the cell.

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