Selective lesion of basal forebrain cholinergic neurons in mice with the mu p75-saporin immunotoxin: Neuroanatomy and behavior

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The basal forebrain cholinergic neurons (BFCNs) are dramatically affected in several neurodegenerative diseases such as Alzheimer’s disease or Rett syndrome. The characterization of the behavioral consequences of selective BFCN lesions is necessary to study the implication of these neurons in cognitive functions. Until recently, this model was not available in mice, despite the growing interest in this species, due to the creation of a wide variety of genetically modified mouse lines modeling neurodegenerative diseases. The first version of a new cholinergic immunotoxin, mu p75-SAP, induced specific lesions of the BFCNs associated with dramatic memory performance deficits, but it also showed side effects and poor survival rates (Berger-

(continued on page 6)

Figure 1. Micrographs of brain coronal sections of mice treated with PBS or mu p75-saporin (SAP). In lesioned mice, the number of ChAT-positive neurons is dramatically reduced in the medial septum (MS) and the diagonal band of Broca (DBB) (a,c) and in the nucleus basalis (NB) (b,d). AChE staining is massively depleted in the cortical mantle (e,g) and the hippocampus (f,h), but not in the thalamus (Th). Scale bar = 200 µm.
ATS Licenses SP-SAP to Advanced Pain Therapeutics

Advanced Targeting Systems is pleased to announce that it has licensed its Substance P receptor-targeted chronic pain drug (SP-SAP). Advanced Pain Therapeutics, LLC (APT) obtained exclusive, worldwide rights to develop, manufacture, use, and sell SP-SAP for the treatment of severe chronic pain. Cato Research, a global contract research and development organization, will provide CRO services to the new company.

SP-SAP is a single-dose, non-opioid, substance P receptor-targeted treatment designed to specifically bind to and eliminate a subset of neurons that send the chronic pain signal to the brain. Preclinical studies in animal models have shown that SP-SAP eliminates chronic pain without disrupting other sensory modalities or motor function and is well tolerated.

“When we partner early, as we have here, we can make a major difference in the overall development of promising drug candidates such as SP-SAP,” said Lynda Sutton, COO of Cato Research and CEO of Advanced Pain Therapeutics. “The flexible, broad-based relationships among APT, ATS, and Cato Research position us well to execute our innovative drug development model.”

Denise Higgins, Vice President of ATS, affirms that this arrangement allows for a unique synergy between the companies. “We are enthusiastic about working with APT and Cato Research to help address the under-served chronic pain population. Our preclinical work with SP-SAP makes us very optimistic about the impact this innovative treatment can have for those who are suffering.”

Chronic disease states and tissue damage can lead to chronic pain. In particular, terminally ill patients often experience severe, chronic pain due to advancement of disease or unwanted side effects of treatment. Although in many cases, standard treatments, such as opioids, can control pain, there is a significant subset of patients who cannot find relief through standard care. Use of opioids can also be associated with unwanted, severe side-effects. In these cases, sedation or cordotomy may be a patient’s only option for pain relief. Hence, severe chronic pain represents a serious, poorly met medical need. SP-SAP offers a novel approach to target a specific set of neurons involved in chronic pain and as such, has the potential to revolutionize the way severe chronic pain is treated.

The solution to the puzzle was:

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   TARGET  S  GROWTH  B  C  E  L  E  T  O  U  T  O  I  N  E
   SP  RECEPTOR  EPA  LEG  FRACTION
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Across:
1. TARGET
5. GROWTH
7. EPA
8. LEG
11. FRACTION
13. TOXIN

Down:
1. TUBE
2. RECEPTOR
3. ERG
4. CAT
6. WELL
9. GEL
10. STUN
12. OIL

Congratulations to the puzzle solvers from our last newsletter. Each winner receives $100 credit towards research product purchases from Advanced Targeting Systems.

WINNERS: Valery Nelson, Panacea Pharmaceuticals, Gaithersburg MD * Barry Marguiles, Towson University, Towson MD * Seto Chice- SUNY HSC at Brooklyn, Brooklyn NY

Experimental Biology
April 5-9, 2008
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Amer Assoc for Cancer Research
April 12-16, 2008
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Booth #1139
Septal grafts restore cognitive abilities and amyloid precursor protein metabolism
Azitria E, Cataudella T, Spampinato S, Leanza G
Neurobiol Aging [Epub Feb 5], 2008.

Although cholinergic loss and the presence of β-amyloid plaques are well documented in Alzheimer’s disease, it is unknown whether restoration of regulatory cholinergic inputs affects in vivo β-amyloid precursor protein (APP). 5 µg of 192-IgG-SAP (Cat. #IT-01) was split between the lateral ventricles of rats. 6 months post-surgery the animals were implanted with cholinergic-rich septal tissue grafts. Grafted animals exhibited normal or near-normal levels of APP. APP levels, as well as improved spatial navigation performance, correlated strongly with graft-induced cholinergic changes.

Selective impairment of the cerebellar C1 module involved in rat hind limb control reduces step-dependent modulation of cutaneous reflexes
Pijpers A, Winkelman BH, Bronsing R, Ruigrok TJ

The cerebellar cortex is arranged in a series of modules. Elucidation of module-specific function has been difficult because of the closely arranged structure of these modules. Here the authors lesioned the C1/C3 hindlimb module of the rat with CTB-SAP (Cat. #IT-14). Rats received 75-125 ng injections of CTB-SAP into the C1 zone of the copula pyramidis or the paramedian lobe of the right cerebellar hemisphere. C1-injected animals displayed marked diminishment of cutaneously induced reflexes with no significant impact on walking or stepping pattern.

The pedunculopontine tegmental nucleus and the nucleus basalis magnocellularis: do both have a role in sustained attention?
Rostron CL, Faquhar MJ, Latimer MP, Winn P

This study provided further investigation into the role of the pedunculopontine tegmental nucleus (PPTg) in control of sustained attention. Rats were given 0.13 µg injections of 192-IgG-SAP (Cat. #IT-01) into the nucleus basalis magnocellularis. The immunotoxin-treated animals were compared to animals receiving ibotenate injections into the PPTg. Results suggest that ibotenate lesions cause impaired selection of conditioned response as shown by an increase in unconditioned behaviors. 192-IgG-SAP treated animals exhibited difficulty obtaining successful lever presses linked to attention.

Spinal mu-opioid receptor-expressing dorsal horn neurons: role in nociception and morphine antinociception
Kline RH, Wiley RG

The authors used Dermorphin-SAP (Cat. #IT-12) to investigate the function of spinal cord mu-opioid receptor (MOR)-expressing dorsal horn neurons in nociception and morphine analgesia. Rats were treated with 500 ng intrathecal injections of Dermorphin-SAP; 500 ng of Blank-SAP (Cat. #IT-21), and up to 1 µg of Saporin (Cat. #PR-01) were used as controls. The data indicate that MOR-expressing dorsal horn neurons are necessary for morphine action and play a role in nocifensive responses to persistent pain in the formalin test.

Basal forebrain and saporin cholinergic lesions: the devil dwells in delivery details
Kalinchuk AV, Porkha-Heiskanen T, McCarley RW

The authors of this commentary discuss results presented by Blanco-Centurion et al. (J Neurosci 26: 8092-8100, 2006). The topic is the role of adenosine in the basal forebrain in the control of sleep homeostasis. Discussion covers the potential differences found when 192-IgG-SAP (Cat. #IT-01) is administered locally as compared to an intracerebroventricular injection.
Targeting Topics: Recent Scientific References

(continued from page 3)

Cholinergic Deafferentation of Prefrontal Cortex Increases Sensitivity to Cross-Modal Distractors during a Sustained Attention Task
Newman LA, McGaughy J

The authors injected 5 ng of 192-IgG-SAP (Cat. #IT-01) into the prefrontal cortex of rats to investigate the effect of cholinergic loss on distractors to attentional demand. Where all animals experienced impaired performance in the presence of visual distractions, lesioned animals were more sensitive to auditory distractions. While these results indicate compromised top-down processing, lesioned animals showed improved performance in bottom-up processing, possibly caused by a shift in circuit dynamics after the lesion.

Effects of ibotenate and 192-IgG-saporin lesions of the nucleus basalis magnocellularis/substantia innominata on spontaneous sleep and wake states and on recovery sleep after sleep deprivation in rats
Kaur S, Junek A, Black MA, Semba K

The caudal basal forebrain of rats was lesioned with 0.26-µg bilateral injections of 192-IgG-SAP (Cat. #IT-01) in order to examine the role of this area of the brain in several facets of sleep behavior. The results suggest that cholinergic neurons and non-cholinergic neurons in the basal forebrain play different, but important roles in non-rapid eye movement sleep and EEG delta power after sleep loss. Non-cholinergic basal forebrain neurons inhibit delta waves, whereas cholinergic neurons promote wakefulness.

Elimination of rat spinal substance P receptor bearing neurons dissociates cardiovascular and nocifensive responses to nicotinic agonists
Khan IM, Wart CV, Singletary EA, Stänislaus S, Deerinck T, Yaksh TL, Printz MP

The intrathecal (IT) administration of nicotinic agonists produces both nocifensive behavior and cardiovascular responses. In this work the authors treated rats with 10 µl of 10-µM SP-SAP (Cat. #IT-07) IT injections; 10 µl of 10-µM Saporin (Cat. #PR-01) was used as a control. Lesioned animals displayed a reduction in response to all nicotinic agonists, but cardiovascular responses to IT nicotine were left intact. The results indicate subunit-specific interactions between the NK-1 receptor and nicotinic receptor systems.

Reactive oxygen species generation by the ethylene-bis-dithiocarbamate (EBDC) fungicide mancozeb and its contribution to neuronal toxicity in mesencephalic cells
Domico LM, Cooper KR, Bernard LP, Zeewal GD

This work explores the mechanisms of neuronal damage associated with the ethylene-bis-dithiocarbamate fungicide mancozeb (MZ). In order to obtain a purified rat mesencephalic culture, the authors treated neuronal cultures with Mac-1-SAP (Cat. #IT-33) at a final concentration of 2 µg/ml. The microglia-free cultures did not display attenuated reactive oxygen species (ROS) production when treated with MZ. The data suggest that microglia are not required for ROS production in the presence of MZ.

Brainstem catecholaminergic neurons modulate both respiratory and cardiovascular function
Li A, Emond L, Nattie E

The authors examined the role of brainstem catecholamine (CA) neurons in various aspects of breathing and chemoreception. Rats received 5-µg injections of anti-DBH-SAP (Cat. #IT-03) into the 4th ventricle; mouse IgG-SAP (Cat. #IT-18) was used as a control. This method of lesioning left the CA neurons in the peripheral nervous system intact. Lesioned animals displayed a constant decrease in breathing frequency, reduced response to CO2, and increased variability of breathing during REM sleep. Inhibitory cardiovascular effects were also seen.

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There are several protocols available.

1. **Preparing for a Cytotoxicity Assay using Secondary Conjugates.** This protocol will be helpful when using our secondary antibody-saporin conjugates with your primary antibody. These include Anti-M-ZAP (Cat. #IT-30), Goat-ZAP (Cat. #IT-22), Hum-ZAP (Cat. #IT-22), Mab-ZAP (Cat. #IT-04), Rab-ZAP (Cat. #IT-05), and Rat-ZAP (Cat. #IT-26).

2. **Preparing for a Cytotoxicity Assay using Streptavidin-ZAP.** This protocol will be helpful when using our streptavidin-saporin conjugate with your biotinylated targeting agent (peptide, ligand, cytokine, growth factor, antibody, etc.).

3. **Concentration Calculation: Convert molarity to mg/ml and mg/ml to molarity.** This protocol will help in determining the correct amount of material to use in your assay. There is also a link to an Online Calculator.

4. **Cytotoxicity Assay for Targeted Toxins in vitro.** This protocol includes photos of what your plates should look like during the assay process. It takes five days to complete this assay. Start on a Monday and develop on Friday. There are many factors that go into a successful cytotoxicity assay. This protocol should help you design and execute appropriately.

5. **Preparing Cytotoxicity Data.** This protocol will give an example of how to process the data from a Cytotoxicity Assay. ATS uses SOFTMax Pro software connected to a plate reader to determine the A490 value. Then we import this data into Prism software (GraphPad) to conduct further data analysis. Here is a figure generated with Prism.

This graph gives important information about how the potency of your targeted toxin. The ED$_{50}$ is the Median Effective Dose (produces desired effect in 50% percent of population). The lower this number is, the more potent the targeted toxin.

We hope these protocols will be helpful to you in your research. If there are additional protocols or tutorials we can provide, please do not hesitate to ask.

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Selective lesion of BFCN with mu p75-SAP

(continued from page 1)

Sweeney et al., 2001; Hunter et al., 2004). Therefore, as soon as the improved version of mu p75-saporin was released by ATS, we characterized the neuroanatomical and behavioral effects of this new cholinergic neurotoxin.

Mu p75-saporin was administered bilaterally in the cerebral ventricles of male C57BL/6J at the dose of 0.4 µg/mouse. At this dose, all treated mice survived and none of them showed abnormal loss of weight or epileptic-like episodes as obtained with higher doses or reported with the previous version of the toxin. The loss of choline acetyltransferase-immunoreactive neurons was more pronounced in the medial septum (82%, see figure 1) and the diagonal band of Broca than in the nucleus basalis (55%). The cholinergic specificity of the lesion was suggested by preserved parvalbumin immunostaining. The hippocampus and several regions of the cortical mantle exhibited a marked drop in the levels of acetylcholinesterase-positive staining. This suggests that septo-hippocampal and basal-cortical projections of the BFCNs underwent massive degeneration. As opposed to the cholinergic toxin 192 IgG-SAP used in rats, mouse Purkinje cells remained undamaged by mu p75-saporin as suggested by preserved anti-calbindin immunostaining in the cerebellum (Traissard et al., 2007). The lesion of the BFCNs affected both locomotor activity as well as learning and memory performances. Nocturnal, and to a lesser extent, diurnal locomotor activity was increased in lesioned mice. The rate of acquisition of a water-maze reference memory task was significantly slower in mice treated with mu p75-saporin (see figure 2). Acquisition performance was also affected in the Barnes maze as suggested by an increase in the total number of holes and in the latency to find the target hole. Retention performance of lesioned mice was lower than those of control mice in both spatial memory tasks, although the effect was significant only in the Barnes-maze probe trial. Motivation, visual capacities and sensorimotor coordination appeared unaffected in the water-maze visual discrimination task and the beam-walking test, respectively.

The lesion of BFCNs with mu p75-saporin induces behavioral deficits similar to those reported after ICV 192 IgG-SAP in rats, but without Purkinje cell damage. In addition, this new version of the mouse immunotoxin has fewer side effects and appears more efficient than its predecessor. This safer and more powerful tool may be particularly adapted to improve transgenic models of AD in which amyloid and/or tangle pathologies are expressed, but do not result in extensive loss of cholinergic neurons.

References

In 2004, ATS re-designed the anti-murine p75-SAP targeted toxin (mu p75-SAP, Cat. #IT-16) and produced a conjugate that is much more potent in our in vitro cell cytotoxicity assays. Previously, we used a rat monoclonal antibody. This antibody had been outperformed by our rabbit polyclonal (Cat. #AB-N01), in several assays, especially flow cytometry analysis of murine p75-expressing cells. This is an important indicator of being able to bind to the cell surface, which is fundamental for a targeted toxin.

To create this toxin, we affinity-purified the rabbit polyclonal (Cat. #AB-N01AP) with the immunogen bound to a solid support, and conjugated the affinity-purified antibody to saporin. As can be seen in the cytotoxicity assay on the right, the new mu p75-SAP is orders of magnitude more potent than the previous conjugate. The new and more active version of mu p75-SAP has an ED50 in the picomolar range compared to an ED50 in the nanomolar range for the previous product. We believe that the greater potency will translate to smaller amounts used for elimination of p75-positive neurons in the mouse brain, and that this will result in a greater index of efficacy and lesser non-specific cytotoxicity. (see cover article for in vivo results).

The mu p75-SAP kit includes, in addition to the immunotoxin, equal aliquots of saporin (Cat. #PR-01), the affinity-purified rabbit polyclonal antibody (AB-N01AP), and the control immunotoxin, Rabbit-IgG-SAP (Cat. #IT-35).

Also available are fluorescent conjugates of AB-N01AP: Cy3-labeled Anti-murine NGFr (Cat. #FL-05), and Cy5-labeled Anti-murine NGFr (Cat. #FL-06).

NG3 cells are plated at 1000 cells/well and incubated overnight. Saporin, mu p75-SAP (conjugate of the affinity-purified rabbit polyclonal to mouse NGFr and saporin), and AB-N02-SAP (previous rat monoclonal version of mu p75-SAP) are added in 10-µl volumes and the plates are incubated 72 hours. PMS/MTS developing reagent is added and the plates are incubated 1-2 hours, then read at 490 nm.

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