REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
20-09-2012	Final Report	1-Nov-2007 - 30-Apr-2009

4. TITLE AND SUBTITLE
Final Report for Controlled Enhancemnt of Long-Term Memory by Modulating Neuronal miRNA Function

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W911NF-08-C-0002

5c. PROGRAM ELEMENT NUMBER
7310AK

6. AUTHORS
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
Science Applications International Corporation-M
10260 Campus Point Drive
San Diego, CA 92121 -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
53406-LS-DRP.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Distribution authorized to U.S. Government Agencies Only, Contains Proprietary information

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The modulation of long-term memory formation in a mouse model has been evaluated following administration of antisense oligonucleotides targeting mRNA from Calcineurin, p250GAP, Armitage, Cbl-b, and CREB-BP in the brain. Alzet minipumps containing stable phosphorothioate antisense oligonucleotides for the 5 targets and a control oligonucleotide were implated in the brains of 51 C57B16 male mice. Following recovery, animals were trained for 3 days, and memory formation was assessed after 3 days, 3 weeks, and 7 weeks. Sustained knock-down

15. SUBJECT TERMS
memory antisense mouse

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Richard Griffey
UL	UL	UL	UL		19b. TELEPHONE NUMBER 858-826-6482

Report Title

Final Report for Controlled Enhancemnt of Long-Term Memory by Modulating Neuronal miRNA Function

ABSTRACT

The modulation of long-term memory formation in a mouse model has been evaluated following administration of antisense oligonucleotides targeting mRNA from Calcineurin, p250GAP, Armitage, Cbl-b, and CREB-BP in the brain. Alzet minipumps containing stable phosphorothioate antisense oligonucleotides for the 5 targets and a control oligonucleotide were implated in the brains of 51 C57B16 male mice. Following recovery, animals were trained for 3 days, and memory formation was assessed after 3 days, 3 weeks, and 7 weeks. Sustained knock-down of Cbl-b statistically enhanced 24 hr and 3 week memory, but produced histological changes in the brains of treated animals.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in peer-reviewed journals: 0.00

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper

TOTAL:

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Received	Paper

TOTAL:

Number of Manuscripts: 0.00

Books

Received	Paper

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
Leon Reijmers	0.20
FTE Equivalent:	**0.20**
Total Number:	**1**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Karsten Baumgartel	0.25
FTE Equivalent:	**0.25**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Mark Mayford	0.10	No
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

1 a. Scripps Research Institute

1 b. 10550 North Torrey Pines Road

La Jolla CA 92037

Sub Contractor Numbers (c):

Patent Clause Number (d-1):

Patent Date (d-2):

Work Description (e): Prof. Mark Mayford performs animal surgery and behavioral testing

Sub Contract Award Date (f-1): 2/22/2008 12:00:00AM

Sub Contract Est Completion Date(f-2): 4/7/2009 12:00:00AM

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1 a. Isis Pharmaceuticals

1 b. 1890 Faraday Ave.

Carlsbad CA 92008

Sub Contractor Numbers (c):

Patent Clause Number (d-1):

Patent Date (d-2):

Work Description (e): Prepare antisense oligonucleotides

Sub Contract Award Date (f-1): 8/1/2007 12:00:00AM

Sub Contract Est Completion Date(f-2): 2/1/2009 12:00:00AM

Inventions (DD882)

Scientific Progress

See Attachment.

Technology Transfer

SAIC Final Report for W911NF-08-C-0002
Controlled Enhancement of Long-Term Memory by Modulating Neuronal RNA Function
1-Nov-2007 to 31-May-2009
Richard Griffey

Abstract
A total of 90 antisense oligonucleotides have been designed and tested in cell culture for their ability to reduce mRNA levels for 5 targets (Cbl-b, CREB-BP, Calcineurin, p250GAP, and Armitage). Lead antisense oligonucleotides were selected based on the level of mRNA reduction at 100 and 300 nM concentrations, and disqualified if any cell toxicity was observed. These compounds were prepared at scale and administered to mice via implanted minipump. An improvement in long-term memory was observed at 3 and 21 days, which correlated with a reduction in Cbl-b mRNA levels in the hippocampus.

Introduction
The genetic factors responsible for long-term memory formation remain poorly defined. While animal models exist to evaluate LTM formation, generation of animals with expression of specific genes knocked out or attenuated has proven time-consuming and difficult. Two approaches for modulation of LTM formation have been described in the literature. The first is facilitation of LTM formation. This has been accomplished through positive modulation of AMPK receptors, and by treatment with ampakines. However, ampakines that accomplish positive modulation have shown undesired clinical side effects. Another approach is to improve consolidation of LTM formation in the brain between 1 and 24 hrs after an experience. This has been demonstrated with modulation of levels of two proteins associated with LTM formation, CREB and Cbl-b. The activity of CREB might be increased with small molecules or levels could be enhanced with inhibitors that attenuate the activity of miRNAs that regulate CREB protein expression. This strategy has pharmacological risk because CREB has multiple functions in brain and other tissues. Alternatively, the level of active CREB could be enhanced by reducing the level of CREB binding protein (CREB-BP) in the brain. Another strategy is to reduce the activity or levels of Cbl-b protein in the brain. The exact biochemical role of Cbl-b is unknown, but it is a protooncogene believed to function as a RING-type E3 ubiquitin ligase that targets signaling proteins for destruction. Cbl-b knock-out mice show modest enhancement of LTM at 7 days.

This study uses antisense oligonucleotides to modulate expression of protein from 5 gene targets in the mouse brain. Antisense oligonucleotides have been designed and tested in cell culture for their ability to reduce mRNA levels of five target genes: Cbl-b, CREB-BP, Calcineurin, p250GAP, and Armitage. Armitage is required for processing microRNAs in all tissues, and it was anticipated that attenuation of Armitage expression might provide insight into the role microRNAs play in modulating LTM formation if the associated toxicity were not too great. The p250GAP protein has been suggested to play a role in inhibiting LTM formation in insects, but the targets of the protein and a

biochemical role in mammalian LTM formation has not been established. Calcineurin is a protein phosphatase that has been linked to neuronal function. Calcineurin is a major calmodulin binding protein in the brain and may inactivate an inhibitor of protein phosphatase-1. It may play an important role in "erasing" short-term memories in the brain before the memories can be strengthened and converted to LTM.

These genes target different steps in the formation of LTM, and were viewed as legitimate targets for antisense compound development to alter LTM formation in the mouse model. The animal model used in the work is not shock-based, but instead uses novel object recognition (NOR) where an observer counts the number of times that a mouse touches objects introduced into the cage in a fixed period of time. Objects are either "old" (prior exposure) or "new" (not seen before). It has been shown that mice will sample a new object more frequently than an old object that they remember from prior exposure. The duration of the memory can be established by exposing the animals to the object set at 1, 7, and 21 days, and comparing the number of times the animal samples the "new" and "old" objects. Equal sampling is taken as evidence that no LTM exists, while lower sampling of the "old" object reflects LTM formation.

In the test protocol, antisense oligonucleotides are administered into one hemisphere of the mouse brain via a cannula attached to an Alzet minipump. The timelines for the

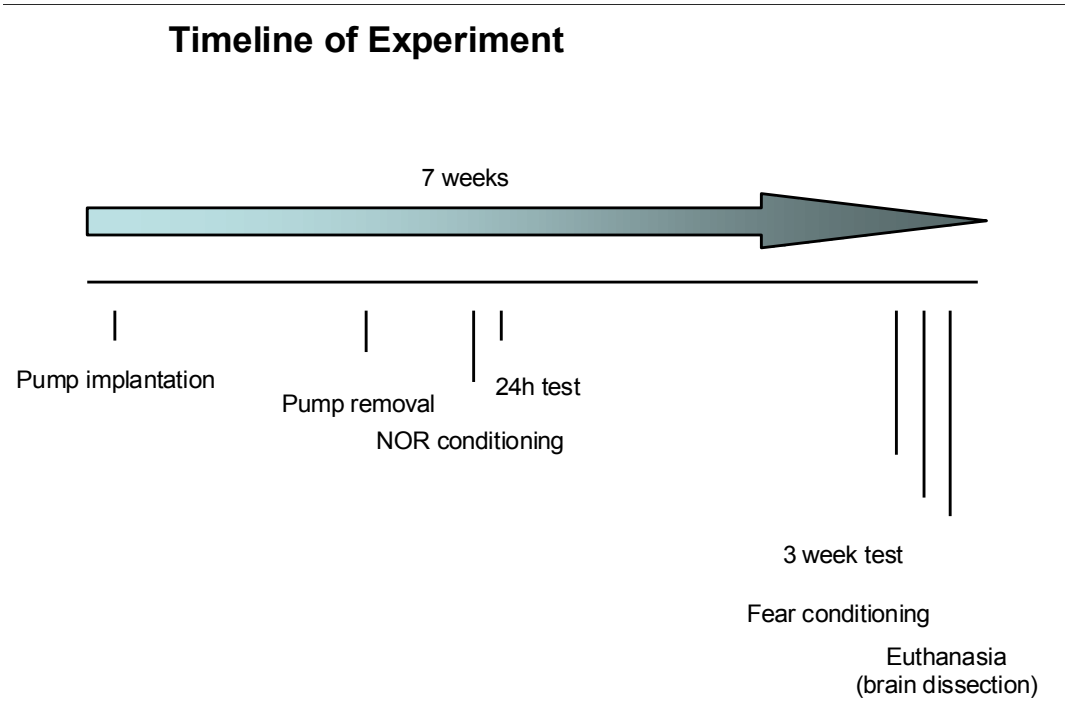

Figure 1. Timeline for animal studies showing minipump implantation, removal, training, and testing. Six groups of 6 animals were studied (control, Calcineurin, Armitage, p250GAP, Cbl-B, and CREB-BP).

experiments are presented in Figure 1. The minipumps are implanted, deliver

oligonucleotide for two weeks, and are removed. Following recovery, training, and testing for memory are performed. Six groups of 6 animals have been studied with oligonucleotides selected for >90% reduction in levels of the target mRNA (control, Calcineurin, Armitage, p250GAP, Cbl-B, and CREB-BP).

Results

The effect of oligonucleotide delivery on general health is presented in Figure 2. Oligonucleotides that provide a sustained reduction in levels of Calcineurin Armitage, and p250GAP mRNAs are lethal or make the animals very lethargic ("sickness" score) and unable to perform simple cognitive processes. The animals receiving the Cbl-b, CREB-BP, and scrambled control oligonucleotides all survived the entire course of the experiment.

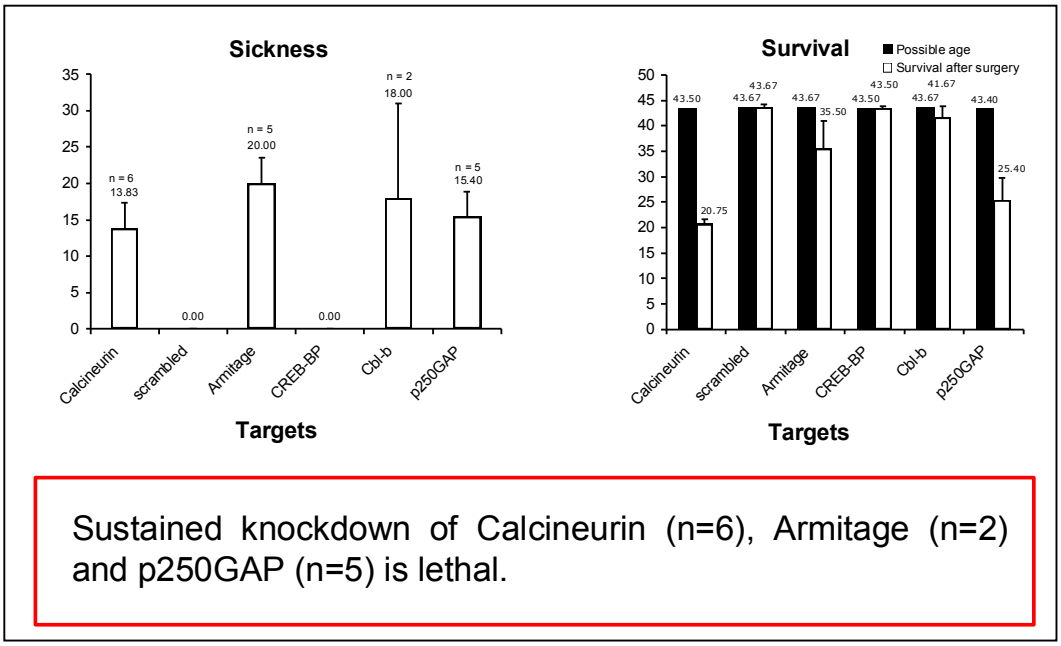

Figure 2. Sickness score and mean survival time for five antisense oligonucleotides and a control oligonucleotide administered to the brain for two weeks via implanted minipump.

The effect of CREB-BP and Cbl-b mRNA knock-down on total object exploration time is presented in Figure 3. Compared to the control oligonucleotide, animals receiving the CREB-BP and Cbl-b oligonucleotide show greater exploration time with objects ("newness") at 24 hrs. However, this effect is lost after 3 weeks, compared to animals receiving the control compound. This demonstrates a potential increase in long-term memory as the objects no longer appear new.

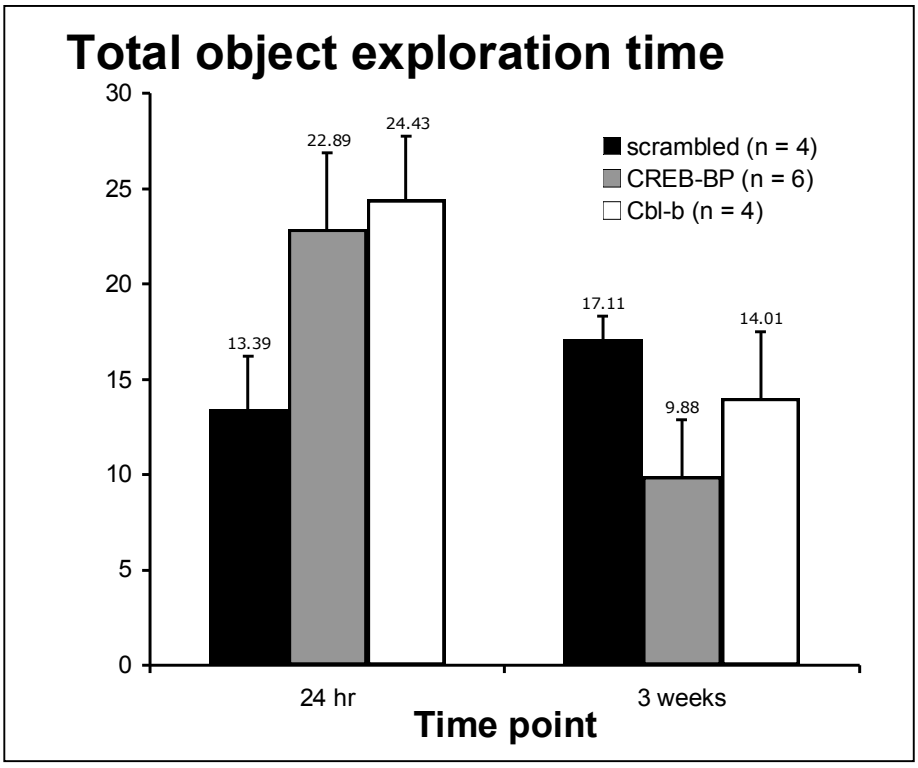

Figure 3. Observed changes in object exploration time after 2 weeks of treatment for control, CREB-BP, and Cbl-b antisense-treated animals at 24 hr and 3 weeks.

The object recognition memory in the first 40 seconds of exploration is presented in Figure 3. The discrimination (time spent exploring the object) between "old" and "new" objects is greater at 24 hrs and 3 weeks in animals with reduced levels of CREB-BP and Cbl-b. As the data is normalized for time spent with the control object, the effect of Cbl-b mRNA knock-down on improving long-term memory is more apparent for the 4 surviving animals.

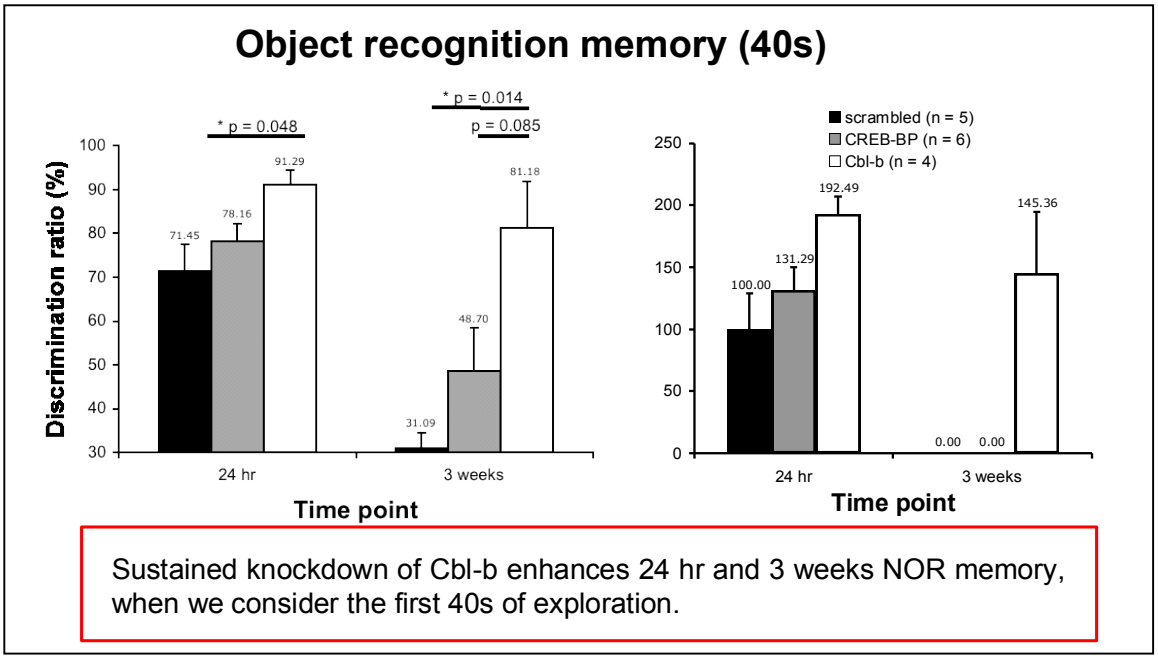

Figure 4. Novel object recognition (NOR) memory at 40 sec is improved in Cbl-b treated animals compared to CREB-BP and control treated animals at 24 hrs and after 3 weeks. Left: absolute discrimination ratio as a function of time spent exploring "new" and "old" objects; Right: percent increase in the discrimination ratio for the "new" versus "old" objects

The effect of mRNA knock-down on fear memory is presented in Figure 5. In this experiment, animals are trained to associate a sound ("cue") with an impending electrical stimulus. Contextual fear memory is initially specific to the cage and timing, but can become generalized over time. The 24 hr contextual memory for the impending stimulation is dramatically increased in animals with reduced Cbl-b, suggesting an increase in short-term memory. The animals are eager to escape the cage, and less freezing behavior is observed until the cue is sounded. The cue induces more freezing in the animals with reduced levels of Cbl-b (Figure 5, lower right) consistent with an increase in 24 hr short-term memory 3 weeks after treatment with the oligonucleotide.

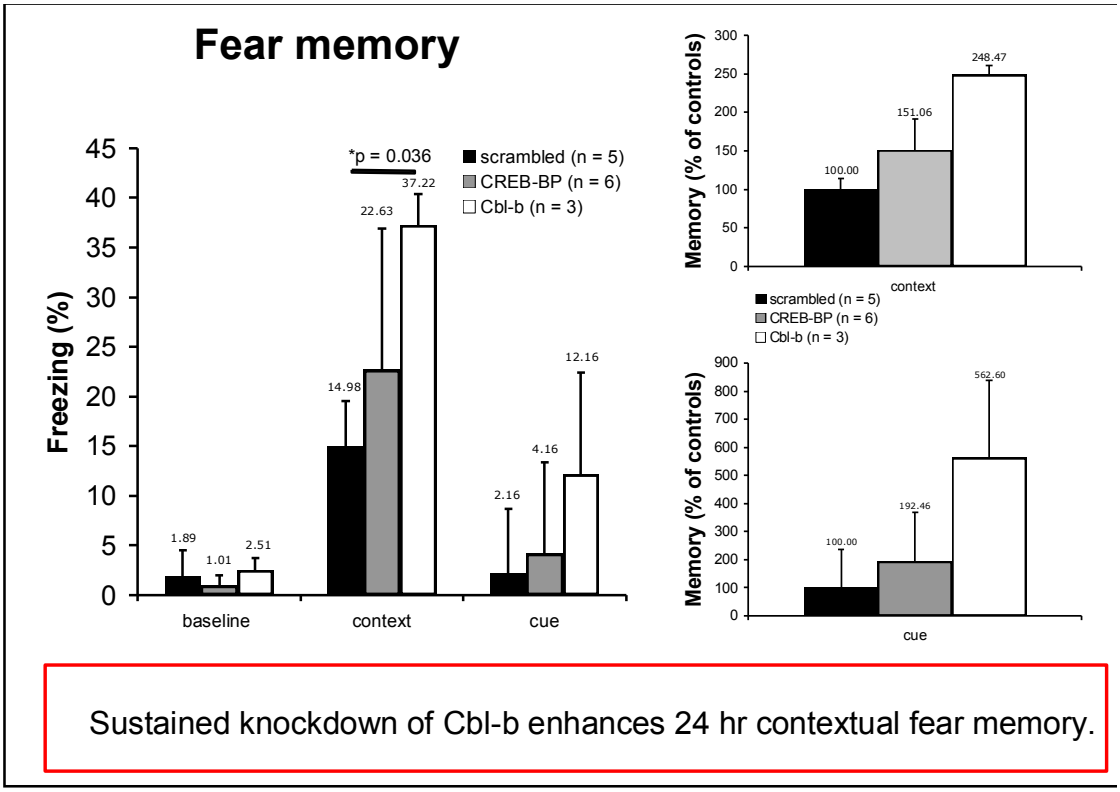

Figure 5. Changes in fear memory 3 weeks following oligonucleotide treatment. Both the context and cue-based freezing response are increased in the treated animals with reduced levels of CREB-BP and Cbl-b. The effect is statistically significant for the Cbl-b treated animals, where a ~500% improvement in cue-based memory is observed.

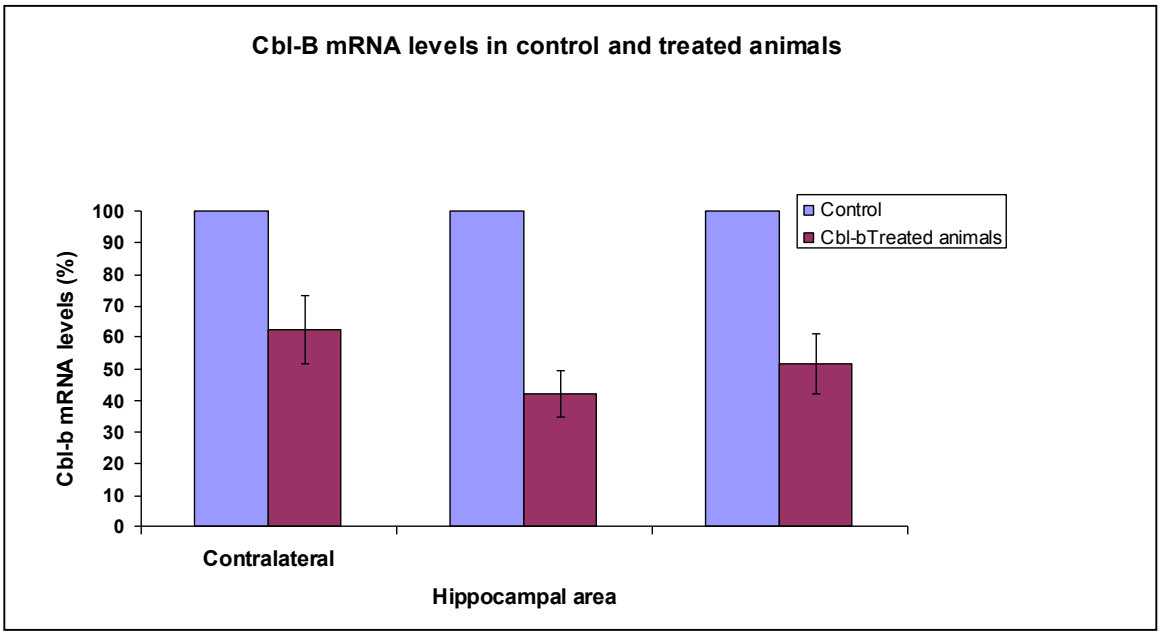

Figure 6. RNA extraction and quantitation of Cbl-b mRNA from hippocampal areas of the brain in control (6) and antisense-treated (5) animals.

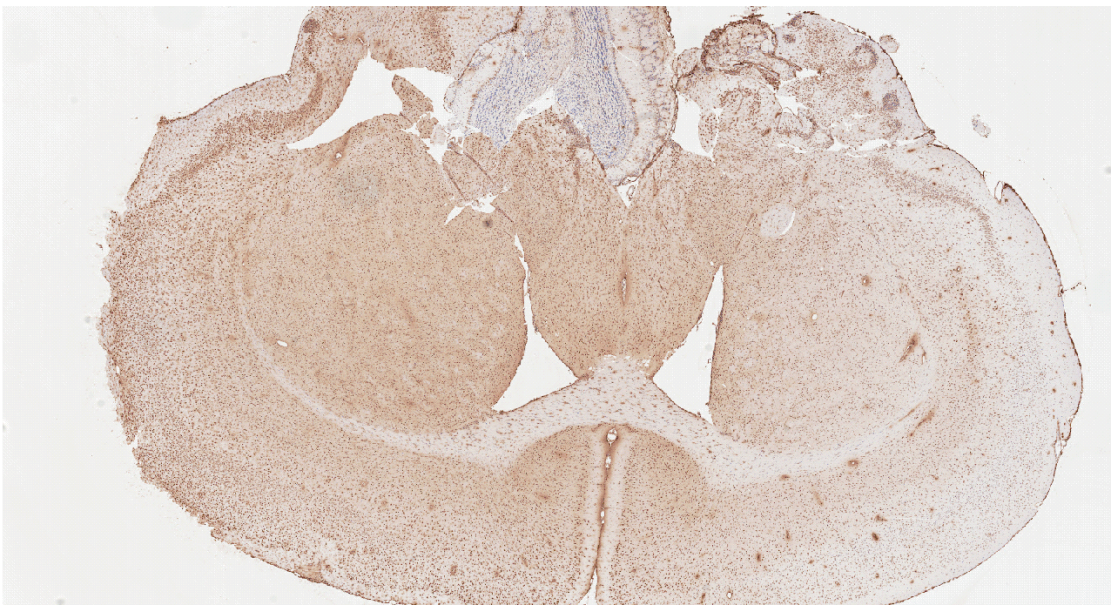

Figure 7. Cbl-b antisense oligonucleotide distribution in the mouse brain. Greater oligonucleotide levels are observed on the left side of the brain and hippocampus compared to the right (contralateral) side of the brain.

RNA Extraction and Quantification of Hippocampal Cbl-b mRNA Levels. Brain tissues from control (6) and Cbl-b (5) animals were sectioned in 2 mm slices of the "anterior" and "posterior" portions of the hippocampus. The anterior section of each brain was

divided into the ipsilateral (same side as the cannula placement) and contralateral sides (opposite to the cannula placement). The posterior section of each brain was placed in formaldehyde for antisense oligonucleotide antibody staining. Samples from both sides were placed in Trizol solution. Tissue homogenization (bead beating) and RNA extraction. RNA levels quantification and normalization. RT-PCR and quantification of Cbl-b mRNA levels relative to GAPDH using the ([Δ][Δ]CT) method. Results are presented in Figure 6. The level of Cbl-b mRNA was reduced by 60% in the hippocampal region of the treated hemisphere, and by 40% in the contralateral hippocampal area. The distribution of the delivery of antisense oligonucleotide to the brain has been determined using an antibody to the "control" phosphorothioate oligonucleotide supplied by Isis Pharmaceuticals and is presented in Figure 7. Greater oligonucleotide levels are observed in the left side of the brain and hippocampus compared to the right (contralateral) side of the brain. While the staining is not quantitative, the qualitative distribution suggests reasonably uniform delivery of oligonucleotide to grey matter, white matter, and hippocampus on the left side of the brain.

Summary

The experiments testing mRNA knock-down in animal models of novel object recognition have shown:

- Two-week treatment with antisense oligonucleotides via implanted minipump produced a 40% reduction in mRNA levels on the contralateral side and a 60% reduction in mRNA in the hippocampal area of the brain. Levels of mRNA reduction are correlated with antisense oligonucleotide levels in the brain.
- Sustained knockdown of Calcineurin, Armitage and p250GAP is toxic and leads to lethargy in surviving animals.
- Sustained knockdown of CREB-BP and Cbl-b increase exploration time for novel objects as compared to control oligonucleotide treatment.
- Sustained knockdown of Cbl-b enhances NOR 24-hr and 3-week memory, while knockdown of CREB-BP has no significant effect.
- Sustained knockdown of Cbl-b enhances 24-hr contextual fear memory, while knockdown of CREB-BP has no statistically significant effect.

The number of animals included in the study were limited, with only 6 animals per group. A repeat of the experiments with Cbl-b target knockdown would provide greater statistical significance for the studies and validation of the target to justify a small molecule drug development effort.