FOTON M-3

Russian - US Collaborative "Receptor II"

Research Proposal by Richard Boyle

Descriptive Title: "Receptor II: Structure and Function of the Gravi-sensing Statocyst System following an Earth's orbital mission."

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Objectives: The "Receptor" project for Foton M-2 was modified by replacing the crayfish experimental model with the snail *(Helix lucorum)* due to biocompatibility problems late in the payload preparation period. The basic hypotheses were not modified, only expanded. This modification had a very significant and positive impact on the success of the project.

During the periods of adaptation to μG and re-adaptation upon return to Earth, it is evident that changes occur within the neural system responsible for transducing and processing the gravity information. We have studied the neural response to transitions between gravity levels with two specific aims:

1. Determine the regulation of expression of the preproHPep gene (gene that is expressed in the primary statocyst receptor cells) as a consequence of space flight and during the re-adaptation to Earth's gravity. Regulation of this gene might signal how the statocyst receptor is ""tuned" by the gravity vector.
2. Specify the cellular response and time-dependent $|Ca^2|$ signaling of the statolith receptors and their influence on the ganglion cells to which they target during the re-adaptation process to Earth's gravity. This combined approach of conventional electrophysiology and optical imaging of somatic Ca^2 measures will provide direct evidence of the cellular events occurring during the readaptation process.

Introduction: After return to Earth many astronauts developed sensations that can be attributed to gravireceptor dysfunction, e.g., illusionary feelings, vertigo, nausea and vomiting, gaze fixation disorder and ocular nystagmus. Our ability to understand the basic mechanisms underlying such dysfunction is limited by our knowledge of the adaptive and re-adaptive responses of the equilibrium organ at the cellular and molecular levels. Adaptation within the gravito-inertial sensing organs can occur rapidly in an organism in direct response to a change in gravitational force (Boyle et al. Neural readaptation to Ig following return from space. *J. Neurophysiol.* 86: 2118-2122. 2001). Our hypothesis is that microgravity challenges the gravireceptors and causes changes in their cellular function, that these changes are manifested in the regulation of specific gene expression and in the electrical behavior of the receptors, and that re-adaptation to 1 g is a time process that can be tracked by intracellular measurements.

We propose to again use the land snail *Helix lucorum* Linnaeus (Pulmonata, Gastropoda) as experimental specimens. These snails are small, strong and can survive unattended with for weeks on a moistened substrate. In addition to Foton M-2. snails have flown on Shuttle. MIR and ISS missions in the past. We continue the M-2 approach for the M-3 opportunity, i.e., we will address our original and extended aims using techniques such as molecular gene expression, intersensory neuronal interactions between the gravi- and photo-sensors, electrophysiology of the voltage and

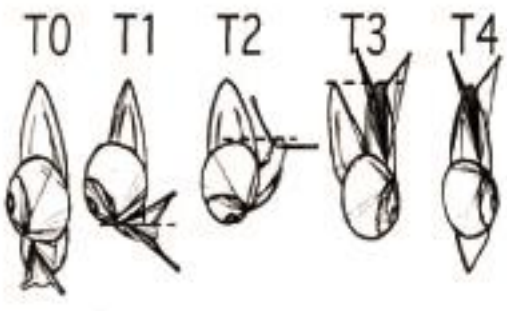
current responses to reorientation with respect to gravity, and optical imaging of the real-time intracellular calcium and voltage signals.

Specify linkage to the NASA Mission:

The basic mechanisms of how living systems sense gravity, transduce the signal and process the information are at the root of understanding how to mitigate problems of orientation, locomotion, and performance both on the Earth, in microgravity and in the fractional gravity environments of the moon and Mars. Behavioral, molecular and electrophysiological measures will be used pre- and post-flight to track changes in organism and afferent/receptor sensitivity.

Hypothesis: The snail statocyst contains calcium carbonate crystals - statoconia - similar to the weight lending otoconia in mammalian otoliths, and the statoreceptors like their otolith counterparts in vertebrates, are activated by changes in animal orientation with respect to gravity. Our hypothesis is that the gravi-sensing receptors are not static sensors but undergo significant structural and functional changes as a result of transitions from one gravity state into another. These changes might be compensatory, but nevertheless maladaptive to the organism that moves into a new gravity state or intermittently along gravity gradients. The initial or adaptive phase could be monitored as the organism transitions into microgravity during flight, but this is technically challenging. The recovery or readaptation of the gravi-sensing statocyst can be experimentally interrogated upon return to Earth's gravity following an orbital mission, and this is our strategy.

Summary of M-2 Findings: The "Receptor I" project evolved into 4 complementary experiments that investigated the influence of microgravity exposure on the structure and function of the gravi-sensing statocyst organ of the snail. The adult snails were studied using two electrophysiology paradigms, and each snail was first behaviorally tested to its response to a sudden 90° reorientation from horizontal to the vertical. These results are designed to put the physiologically measured findings into a behavioral perspective. In the initial test the behavioral "negative gravitaxis" responses of the snails showed that the flight snails in general responded faster than their control counterparts. Figure 1 shows the test procedure: phases 1-4 represent the epochs of behavioral response following a horizontal to vertical pitch of the snail. Figure 2 plots of the time of the behavioral responses at the separate 4 phases for the negative gravitaxis response for the 15 flight and 8 control snails. Flight snails were faster (shorter latency) in their response to pitch stimulation at each phase, and this became significant ($p < 0.05$: nonparametric. two-tailed, unpaired Mann-Whitney test) at phases 3 and 4.



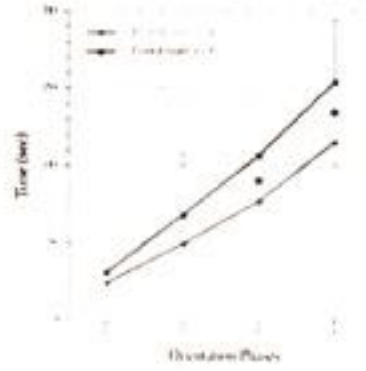


Figure 1 Figure 2

In the morphology study 20 juvenile snails were studied at two time periods following landing.

Examination of the regulation of expression of the preproHPep gene was performed on post-flight and control snails using mRN'A expression techniques. This pedal peptide is found in the primary statocyst receptor cells, and the regulation of this gene might signal how the statocyst receptor is "tuned" by the gravity vector. Compared to controls, flight snails have a higher level of expression in statocyst ganglia but not in other ganglia.

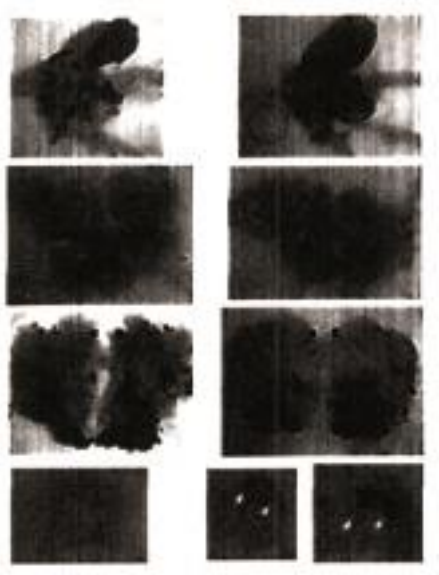


Fig. 3: Pedal peptide gene expression in snail nervous system.

Figure 3 shows light microscopic images of the preproHPep gene expression in 4 separate regions of the snail nervous system. From top to bottom are: cerebral ganglia subesophageal ganglia complex, pedal ganglia, and the *statocysts.* The control tissue is on the left and the flight sample in on the right. *Note the 2 labeled siatoreceptors labeled in the flight snails (arrows).* No significant difference was observed in the other tissue regions between the control and flight samples. suggesting that the upregulation of the gene expression to the pedal peptide was specific to the statocyst. Results of in situ hybridization with probes to the pedal peptide snail gene are given in Table I.

Table 1:

Conditions	# snails	# statocysis	# statocysts with stained neurons
24 h post-flight	6	12	6(4L. 2 R) stained. 2 neurons in each. Rostral part of statocyst
36 h post-flight	6	12	4 stained (2 L. 2 R). 2 neurons in each. Rosiral part
Control	12	24	No stained neurons
Naive snails	10	20	No stained neurons
Naive 24 h after foot cut	4	8	In 1 statocyst onl) 2 stained neurons

Naive 24 li after centrifuge	7	14	No stained statocysts

The two separate electrophysiology experiments were conducted to study the readaptation of the statoreceptor and orientation systems following exposure to microgravity and return to lg. A tremendous amount of data was collected from the whole nerve preparation and individual statoreceptors in these experiments, and we are still analyzing the data and compiling the results. At this moment we can state the following findings. 1) Intersensory interaction between the photosensors and the gravisensors is altered. A significant difference was observed in the effect of light stimulation on the background firing rate of statoreceptors in control and post-flight snails (Fig. 4). In addition, extracellularly recorded neural responses of the statocyst nerve to adequate motion stimulation in the post-flight snails were independent of the motion direction while in the control animal significant differences in responses to different directions were observed. Further, a high significant difference in whole nerve activity responses before and after rise and fall of the test platform was seen. The simplest explanation of these results is that the snail loses preferential direction (i.e. orientation) during flight, and this is reflected in the measured responses in the intersensorv interaction tests.

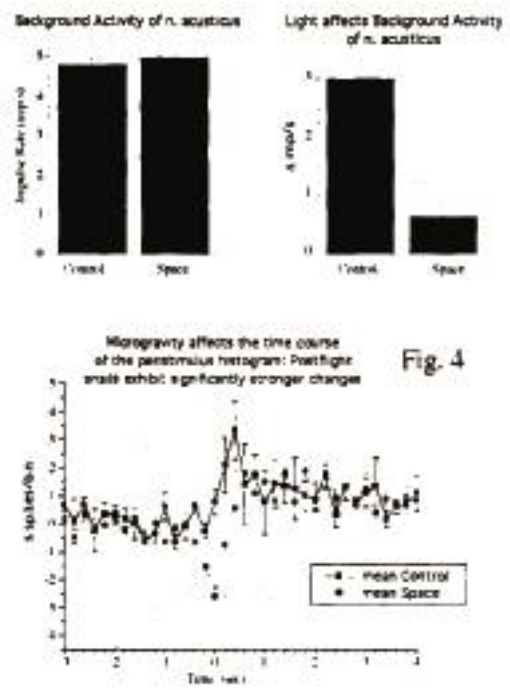


Fig. 4

2) The intracellular response sensitivity of individual gravi-receptors to natural tilt/pitch stimulation was measured using electrophysiology (bridge and clamp amplifier: npi Germany) and real-time voltage- and calcium-sensitive dyes imaging techniques (high-speed fluorescent microscopy and RedShirtlmaging software). Salt-free solutions were used in some experiments to query the role of sodium and calcium ions in shaping the voltage and current responses of the statoreceptors to tilt in post-flight and control snails. The inward sodium current drives the action potential, but the results are still being evaluated and discussed. Although the sample is small, the results support the hypothesis that the statoreceptor sensitivity is altered -an upregulation - by the exposure to microgravity. This agrees with the evidence in vertebrate species (fish, rats, monkeys and humans) that an initial phase of hypersensitivity occurs in the otolith organ. The results are

presented in Figs. 5 and 6. Figure 5 shows the current response under voltage clamp conditions of the statoreceptors. The membrane potential was held near rest in control (red trace at -57 mV) and in post-flight (blue trace at -50 mV) statoreceptor. The inward current (downward movement of the current trace) in response to a 10° tilt of the animal in its preferred plane was more pronounced and showed less recovery or adaptation during the stimulus in the post-flight snail. Figure 6 shows the results obtained using calcium imaging techniques. Two individual statoreceptors were intracellular^ labeled with Oregon Green indicator dye (enhanced image on right side) and the measured calcium signals are shown for one cell on the left. In response to a 10° tilt the cell showed a 3 fold increase in voltage activated calcium spikes (associated with the action potential) and an enhance calcium conductance.

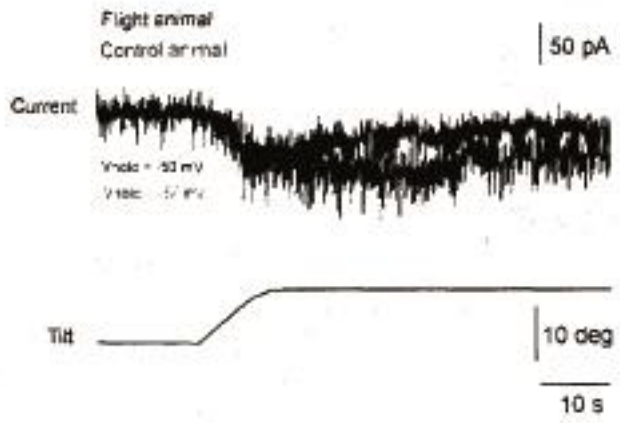


Fig. 5: statoreceptor current responses

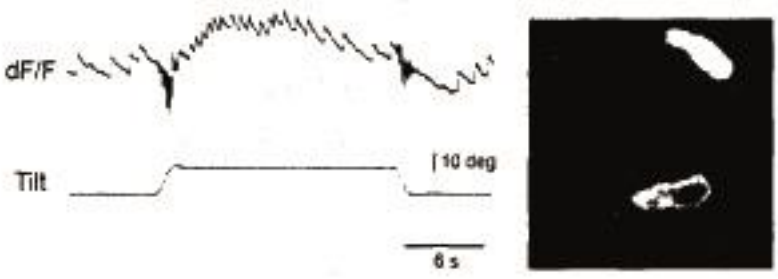


Fig. 6: calcium signaling and imaging

Abstract of Foton M-2 Report:

27lh Annual International Gravitational Physiology Meeting 23-28 April, 2006 Osaka University, Osaka, Japan

STRUCTURE AND FUNCTION OF THE SNAIL STATOCYST SYSTEM AFTER A 16-DAY FLIGHT ON FOTON-M-2. P. M. BALABAN, A. Y. MALYSHEV, I. S. ZAKHAROV, N. A. ASEEV, N. I. BRAVARENKO, V. N. lERUSALIMSKY. A. I. SAMAROVA, D. VORONTZOV, Y. POPOVA*, R. BOYLE* Institute of Higher Nervous Activity and Neurophysiology. Russian Academy of Sciences, Butlerova 5A, 117485. Moscow, Russia and *NASA BioVIS Center, Ames Research Center, USA.

In terrestrial gastropod snail *Helix lucorum L.* we studied the changes after a 16-day exposure to microgravity in: behavior, neural responses to adequate motion stimulation. intersensory interactions between the photosensory pathways and the statocyst receptors, and in expression of the HPeP gene in the primary statocyst receptor cells. In behavioral experiments it was found that the latency of body position change to sudden orientation change (flip from horizontal to downwards position) was significantly reduced in the postflight snails. Extracellularly recorded neural responses of the statocyst nerve to adequate motion stimulation in the postflight snails were independent of the motion direction while in the control animals significant differences in responses to different directions were observed. It was possible to distinguish responses of up to 11 primary receptor neurons from the 13 that comprise the statocyst in the snail. No effect of light (intersensory interactions) was observed in postflight snails in the statocyst receptor responses, while in control animals the presence of light significantly modified the responses to different directions of motion. The HPeP gene peptide product is known to be involved in cilia beating control in the snail. Significant differences in the HPeP gene mRNA expression pattern in the statocyst receptor neurons were observed in postflight (24h) and control snails, thus supporting the observed postflight changes in receptor responses to gravitational stimuli. Obtained results confirm the possibility to elucidate the influence of microgravity exposure on subcellular structural mechanisms and function of gravireceptors using this simple model animal.

Plans for Foton M-3 Mission "Receptor II"

The basic experimental design developed for M-2 will be followed for M-3 experiments. A major obstacle that all space biologists face in publishing microgravity results obtained from orbital missions in the best journals is the fact that confirmation of findings is often not possible - the study essentially represents an "n" of one. The M-3 project will be an important contribution to the scientific conclusions reached from M-2, and will allow not only a confirmation of the M-2 results, but also an improvement of techniques and expansion of the study. The overall goal is to understand the influence of altered gravity, such as microgravity, and transitions between low to higher level, on the gravi-sensors.. The M-3 studies are important steps in reaching this goal. The snail has proven to be a robust model, easy to maintain, and possesses a nervous system and gravi-sensing statocyst organs that are responsive to the space environment. The Russian members are experts with this model organism, particularly in the field of learning and memory. The animal habitats were successfully flown on M-2 and provided an adequate environment for both juvenile and adult snails despite the limitations of the Foton satellite. Importantly, the snails were active during the mission, and only one adult snail went into a hibernating state. Thus, a successful experimental study on M-3 is not in doubt. We also assume that the return of the animals from M-3 to the host labs will be significantly improved by the modification of the M-3 for a controlled (thrust capabilities) re-entry and a landing at a Russian site. This would be in itself a *significant* and *an important* enhancement of the projects. Neural readaptation in vertebrates begins immediately upon return to the atmosphere and is well underway if not virtually complete in about 30-48 hours post-flight. In snails it appears to be delayed as evident from our M-2 results. The early return of the animals to the lab would permit more thorough and extensive experimentation, and the examination of the specimens in the more critical hours after landing.

All experiments are conducted at the Institute of Higher Nervous Activity and Neurophysiology. Preflight control experiments are performed to verify the experimental protocol such as sequencing of tests, correct if necessary technical or instrumentation issues, and sharpen the skills. Experiments continue during the flight as needed to establish baselines and benchmarks. Post-flight experiments are conducted, followed by the delayed (2-3 days) flight temperature control experiments. Preliminary survey of the data is made with the entire science team in Moscow, and the thorough data analysis will be performed in Moscow and at NASA Ames.

M-3 snails will be separated as before into two groups: juvenile snails for the antibody and gene expression labeling studies and the adult snails for the behavioral and electrophysiological

experiments. The gene expression techniques have worked as expected in the Russian lab, and they now have the antibody staining techniques perfected (the antibody labeling was unsuccessful at the time of the M-2 landing). The M-3 experiments will be performed to improve the reliability (see Table 1) of the M-2 results and extend the antibody labeling for functional verification of the upregulation in petal peptide gene expression. We are currently exploring the option to look for other peptides expressed to reveal the specificity of different statocyst neurons. It is thought that each of the 13 neurons has a specific intracellular signature, as in a mosaic arrangement of different neurotransmitters. In this case immunocytochemistry techniques will be used on one of the pair of statocysts. Along the same lines we will determine whether this specificity can be linked to intracellular interconnections between individual cells before/after the flight using intracellular recording or using real-time intracellular calcium imaging techniques. The additional behavioral experiments from M-3 are needed to quantitate the changes in negative gravitaxis response (see Figs. 1 and 2). In addition these tests take little time and provide a behavioral reference from which to evaluate the other results. We are discussing adding another paradigm to measure both the speed of behavioral response to negative gravitaxis with a positive response to chemical stimuli (acetone/carrot). Differences here might reflect functional disturbances of the spatial orientation system. The results of inter-sensory interaction between gravity- and photo sensor showed that there is significant difference in effect of light on background firing rate in control and post-flight snails, but differences in the effects of light on normalized responses in control and post-flight animals are absent! The M-3 data will validate these observations. We will repeat the voltage recordings and calcium signaling in individual receptors to tilt stimulation. It is essential that we obtain a larger sample of statoreceptors recorded simultaneously. We are currently testing the non-toxic delivery mechanism by Gene Tools (Endo-Porter) to transport the dye into the entire population. If successful, then the entire statoreceptor pool can be tested simultaneously to the natural tilt stimuli shortly after return. If the delivery mechanism proves less than reliable in control studies, we will intracellularly label using the glass microelectrode (proven technique, see Fig. 6) in quick succession up to 6-8 individual statoreceptors in the M-3 snails.