UNIVERSITIY OF CALIFORNIA SAN DIEGO

Identifying Molecular Determinants Responsible for Motor Learning.

A thesis submitted in partial satisfaction of the requirements

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by

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The Thesis of Shawn Putrus is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATIONS

To my unconditionally loving mother, Sundus, my hard-working father, Mazin, my ambitious brother, Luke, and my loving dog, Teddy, I would not be where I am today without your guidance and support. Thank you for taking part in this journey and setting an example of what it means to work hard and persevere in all facets of life.

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ABBREVIATIONS

FRT	Forelimb Reach Task
APP	Amyloid Precursor Protein
sAPPα	Secreted APP alpha
CST	Corticospinal tract

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ABSTRACT OF THE THESIS

Identifying Molecular Determinants Responsible for Motor Learning.

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Fine motor behavior and circuit development of the motor cortex is associated with selective structural expansion in networks of functionally related neurons [1]. Corticospinal neurons are amongst these that exhibit extensive structural plasticity when subjected to forelimb-specific behavioral tasks where these neurons undergo elaborate enrichment in dendritic boutons and an increase in apical and basilar spine density. In contrast, neighboring neurons not directly engaged in the motor behavior do not display the same structural modifications as a consequence of learning. We wanted to determine what drives this anatomical change on a molecular level. First, we identified the learning transcriptome by isolating C8-projecting corticospinal neurons that were subjected to skilled grasped training and purified mRNA-associated ribosomes. Our RNA sequencing revealed amyloid precursor protein as a functional network hub associated with motor learning. We determined if APP protein is upregulated in response to motor learning. Animals were subjected to either 1-day or 7-days of forelimb-skilled grasp training, motor cortices were isolated, and protein extracted. Western blot analysis indicated that holo APP protein is slightly upregulated in response to learning compared to untrained animals. APP proteolytic fragments showed a more robust upregulation in response to learning, and histology of these animals showed APP localization within layer 5 motor neurons suggesting APP protein plays a role during learning where this protein is proteolytically cleaved in response to this task.

INTRODUCTION

Motor Learning

Motor learning is a complex process categorized into several stages. First, skilled performance requires the efficient and correct sensory input, such as visual cues to orient the subject spatially, and the ability to extract task-relevant information. Secondly, a motor-driven decision is made based on this sensory input to execute the task. When a novel skilled motor task is learned, basic mapping rules are first explored between eye-hand coordination and target-hand locations which are initially coded as vectors transformed into a motor command [2]. As hand-motor commands and visual-sensory inputs are coordinately activated the subject will undergo adaptation, where the modification of movement from trial-to-trial is based on error feedback in which the following criteria are met [3]:

1.The movement contains a specific action, such as reaching, and can change in terms of force and direction.

2. Adaptation occurs with repetition of the behavior and is gradual over time.

3. Once adapted, subjects cannot revert back to the prior state, but to de-adapt subjects must lose the skill with practice in the same gradual manner to revert back to the original state.

Lastly, a consolidation period solidifies the learned state where reorganization of new representations is “saved” in performance. This process makes motor memory distinct from declarative memory, which can recall a single item through single-trial memory, such as remembering where you parked your car today. Motor memory in contrast requires repetition but once acquired has the capacity to form long-term memories, as we don’t forget how to ride a bike or how to swim once learned. Here we

took advantage of skilled forelimb reach task in rodents to investigate the molecular changes associated with motor learning.

Skilled Forelimb Reach Task

Skilled reaching movements are an important aspect to human motor skill and behavior. It has been widely used as a means to study damage to the motor system after stroke and in neurodegenerate disorders such as Parkinson's disease [4]. Understanding this complex behavior on a molecular level will provide mechanistic insight not yet realized that may prove beneficial for patients who have lost this vital motor skill. We utilized a mouse model of skilled reaching grasp that involves correct orientation to the target. As the paw pronates over the food, the tips of digits 2 through 5 are placed onto the target in an arpeggio movement. A downward movement of the palm palpates the target and if food is not successfully engaged, the paw is withdrawn without grasping. If food is contacted, the food is manipulated and grasped by the digits with a grasp pattern and withdrawn to the mouth [5]. This dexterous utilization of the hand digits to grasp is developed in rodents and not a natural skill previously acquired [6], which can be measured quantitatively over time.

In our studies, we used this skill to measure molecular changes associated with motor learning, and further identified key molecular determinants that drive the acquisition of the task, or the performance acquired. A key motor circuit involved in forelimb motor function is the corticospinal tract. These corticospinal neurons reside within the cerebral cortex and extend their axons along the spinal axis where they terminate onto lower motor neurons and interneurons of the spinal cord, controlling movements of the limbs and trunk.

The Corticospinal Tract

The corticospinal system has been previously shown to play a role in forelimb motor function and undergo circuit adaptation in response to learning [1; 7]. Plasticity of a circuit often involves the formation of new synaptic structures such as dendritic spines and synaptogenesis where the formation of new synapses can undergo synaptic pruning[8]. For instance, when corticospinal layer V neurons that project into the C8 level of the spinal cord were engaged in a new motor task, such as forelimb grasping task, a 22% increase in dendritic apical spines were observed [1]. Conversely, the layer V corticospinal neurons that project into the C4 level of the spine that are not involved in the motor task, but innervate the proximal forelimb, shoulder and neck showed no change in spinal number nor dendritic complexity. This evidence conveys that only a specific population of neurons involved in a task undergoes structural changes in response to learning [1].

This plasticity was further attributed to a 2.5-fold increase in recurrent circuit connectivity among neurons in layer 5 that is specifically engaged in the task [7]. These same corticospinal neurons display an increase in excitability following learning, suggesting a reorganization of the recurrent motor network. Here we wanted to understand the molecular changes associated with these anatomical changes.

Synaptic Plasticity

Increases in spine density and synaptic connectivity rely on synaptic plasticity. The initial phases of plasticity begin with the release of glutamate from the presynaptic terminal. Glutamate diffuses across the synaptic cleft in order to bind to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors located on the postsynaptic terminals. Subsequently, AMPA receptors are activated and NMDA receptors are inactivated through a magnesium

receptor blocker. Once Na^+ and K^+ enter the cell via the AMPA receptor, the postsynaptic neuron will depolarize. This causes the removal of the magnesium blocker, which allows Na^+, K^+ and, most importantly, Ca^{+2} to enter the cell. This series of events allows more AMPA receptors to be incorporated into the postsynaptic membrane that keeps the synapse strengthened and increases the excitatory current size [9]. The second phase of plasticity deals with memory acquisition. Acquisition has to do with the establishing of new memories, and it also follows the same mechanism of NMDA/AMPA explained previously. With regard to motor skill acquisition, the VTA projects into the primary motor cortex (M1) and releases dopamine that will eventually lead to gene expression and plasticity [10]. The final stage of plasticity, known as memory consolidation, is when memory is converted from immature or fragile to mature or permanent. One example of this process can be seen in studies in Aplysia sensitization of gill-withdrawal reflex. When a mild shock to the tail is given, short-term sensitization is seen and lasts for minutes. If five shocks given in a temporal manner to the Aplysia's tail, long-term sensitization could last for several weeks.

Two types of structural changes were associated with long-term sensitization. First, the preexisting presynaptic compartment underwent structural remodeling. This remodeling caused an increase in the size and number of the vesicles that released neurotransmitters specifically in the sensory neurons of trained animals in comparison to untrained controls. Secondly, a two-fold increase in synaptic varicosities (boutons) and larger synaptic arbors were seen in these sensory neurons that were associated with trained animals when compared to untrained animals [11].

Comparing these findings to motor learning, we have similarly observed an increase in dendritic complexity of apical and basilar spines of corticospinal neurons that undergo motor learning, compared to untrained controlled subjects [1]. We further

observed greater network expansion and connectivity between C8-projecting corticospinal neurons that project to the proximal forelimb associated with the task, compared to untrained animals [7]. Interestingly, this increase in synaptic connectivity was only observed between C8-projecting neurons, but not between C4-projecting neurons suggesting this network expansion and connectivity increase is directly related to motor learning. These findings propelled us to investigate the molecular changes associated with learning.

APP in the context of learning

Amyloid precursor protein (APP) has been implicated in Alzheimer pathogenesis, but its role within the non-diseased state is not well understood. APP is proteolytically cleaved by either α-secretase or β-secretase, followed by γ-secretase, resulting in two principal physiological pathways: the amyloidogenic pathway or the non-amyloidogenic pathway. In the event that α-secretase cleaves first, considered the major pathway [12] where the cleavage site of this metalloprotease resides within the Aβ sequence, the non-amyloidogenic pathway is favored leading to the secretion of sAPPα, which has been associated with synaptogenesis, memory enhancement, and neuroprotection [13]. When β-secretase cleaves first, by subsequent γ-secretase cleavage, the amyloidogenic pathway is favored producing Aβ plaques associated with Alzheimer's pathology.

Both α-secretase and β-secretase cleavage of APP results in an extracellular domain released, called sAPPα (83.4 kDa in size) and sAPPβ, respectively. A number of studies have suggested a trophic role for sAPPα [14]. In patients with Alzheimer's disease, there is a lower concentration of sAPPα in their cerebrospinal fluid compared

to healthy subjects [15]. Low concentrations of sAPPα has been correlated with a deficit in spatial memory performance, neuronal survival and deficit in hippocampal-mediated memory [16]. The neurotrophic effects of sAPPα includes its ability to reduce neuronal cell death in neurons that are under stress (e.g. UV radiation and hypoglycemia) [12].

Interestingly, sAPPα has been implicated in memory consolidation. Studies on neuronal cells have shown that APP can promote cell growth and that a peptide containing the neurotrophic domain of sAPPα increases the number of synapses in the cortex during learning [17]. The number of synapses and endogenous APP at synapses in the cerebral cortex increased when rats were placed in enriched environments [18]. sAPPα has also been shown to enhance long-term potentiation and modulate the induction of long-tern depression in hippocampal slices [19], suggesting APP can act on synaptic events underlying memory processes.

More recent work has shown that in mice that lack the full length APP (conditional knockout) and the compensatory protein APP like protein 2 (APLP2, conditional knockout) displayed a loss in basilar and apical dendritic length and complexity of CA1 pyramidal hippocampal cells [13]. These subjects had a deficit in spatial learning, escape latency, and swim path length compared to control subjects who did not receive a double conditional knockout of APP and APLP2. When sAPPα was supplemented to the double conditional knockout subjects, a significant improvement was seen in behavioral performance, as well as a restoration of dendritic complexity of pyramidal hippocampal cells. Contrary, when sAPPβ was supplemented into the double knockout, there was no increase in behavioral performance compared to controls, and no change in spine density or complexity. This suggests a direct role of sAPPα in hippocampal mediated memory and learning.

The intracellular proteolytic fragment products of APP are known as intracellular domains (AICD). These 5 kDa peptides functions in cell signaling by binding to transcription coregulators and adapter proteins. One such adapter called Fe65 has been shown to play a role in diverse cellular functions [20]. Fe65 has been shown to stabilize AICD prior to translocation into the nucleus, where it regulates gene expression. Once inside the nucleus, it complexes with CP2/LSF/LBP and Tip60 to eventually activate glycogen synthase kinase-3 (GSK-3β) involved in neural development [14; 21].

In summary, the cascade of molecular events underlying the neuromodulatory properties of APP are still to be elucidated, and the direct role of APP in motor learning is yet to be identified. The present body of work suggests APP plays a role in motor learning.

RESULTS

Phases of Motor Learning

In previous studies we have shown that motor learning is associated with selective structural changes in networks of neurons that are functionally related to the task, and that distinct C8-projecting corticospinal neurons undergo increased dendritic complexity with a 2.5-fold increase in connectivity with one another in response to motor learning. Here we performed forelimb reach task, and isolated motor cortices that were intersectionally labeled with GFP-positive ribosomes. To achieve this, an AAV9-CAMK2-CRE viral vector was injected into spinal cord cervical level 8 and retrogradely transported to the motor cortex. Two weeks later, AAV8-Ribotag-GFP$^{fl/fl}$ was injected into the motor cortex where the presence of cre-recombinase would recombine to express a GFP tag on ribosomal protein L10a. Immuno-precipitation against GFP would then pull down only GFP-tagged ribosomes, and mRNA isolated from that precipitate fraction to identify the actively translating transcriptome. Three weeks later, animals were subjected to handling, followed by forelimb reach task.

We isolated the learning transcriptome at 1 day after learning, as this identifies an early time point where learning has only been initiated but not achieved. At this time point, animals are only 10-20% successful in the task of forelimb reach. Our next time point was 4 days of learning where animals are on average 50% successful in learning, and a rapid learning curve has been achieved. This would constitute the rapid learning phase. We next isolated the learning transcriptome at 7 days after learning where success rate is 60-70%. This is the most success subjects will achieve in this task, and constitutes the completion of learning. We further isolated the transcriptome at 14 days after learning to capture the consolidation of learning. This represents the process by

which immature synaptic connections are strengthened towards a permanent state through the repetition of the behavior task.

We accordingly characterized the transcriptional state of these 4 stages of learning (1, 4, 7, and 14 days of learning) to represent states of initiation of learning, rapid learning, learned phase, and memory consolidation, respectively.

The learning Transcriptome

The learning transcriptome of C8-projecting corticospinal neurons showed a distinct pattern of gene expression over time as these animals engaged in the task and acquired the skill, FDR<0.1 (Fig 1A). Principal component analysis indicated a clear shift of gene expression along two main eigen vectors as animals learned (Fig 1B), where 939 gees were upregulated at day 4, 1009 genes were upregulated at day 7, and 2122 genes were upregulated at day 14 of learning compared to intact ($p<0.05$). Contrary, 217 genes were downregulated at day 1 of learning, 692 genes were downregulated at day 4, 847 genes were downregulated at day 7, and 1946 genes were downregulated at day 14 of learning. Network analysis revealed APP as a central hub at all time points ($p = 0.01$ at day 1; $p = 0.001$ at day 4 and day 7; $p = 0.000001$ at 14 days) using ingenuity pathway analysis (Fig 1C). APP was further identified as a top upstream regulator along all time points (Fig 1D), suggesting APP may play a role in motor learning. We wanted to investigate the role of APP in the context of motor learning.

APP Protein levels in response to Motor Learning

To determine if APP protein is upregulated after motor learning and/or alternatively spliced in response to learning, animals were trained in forelimb reach task and protein isolated from the motor cortex at 0 days, 1 day, and 7 days after

learning (Fig 2A). Western blot analysis was used to determine if APP protein levels are altered in response to learning compared to untrained subjects. Using an APP antibody 22C11 that targets the N-terminus of the extracellular domain, holo APP levels showed a slight increase in protein expression at 7 days of learning compared to untrained control animals (Fig 2B,D). This APP fragment detected may represent total APP protein (100 kDa), sAPPα (83.4 kDa) or sAPPβ (31.4 kDa). Because antibody 22C11 resides on the N-terminus of the protein, the exact species detected cannot be determined using this methodology only. Additional studies will have to be conducted to determine the exact nature of the APP products in response to learning.

To determine alternate APP proteolytic processing, we used an antibody directed against the C-terminus of APP, Y188 that binds to the endocytosis domain of the protein. APP cleavage by β-secretase occurs within endocytic vesicles, and not on the plasma membrane. Internalization of APP is therefore critical for amyloidogenic mediated APP processing. Western blot analysis using Y188 showed a protein fragment migrating at 20 kDa. This fragment incrementally increased with learning, where a significant increase was observed at 7 days of learning (Fig 2B,D). Although western blot analysis does not reveal the specific identity of these peptides, future studies that includes mass spectroscopy will need to be performed to identify the exact APP proteolytic fragments produced in response to learning.

APP Expression in Corticospinal Neurons

To determine if APP protein is expressed in C8-projecting corticospinal neurons, a retrograde virus (AAVrg) was injected into the grey matter axon terminals at cervical level 8 of the spinal cord. Animals were then subjected to forelimb reach task

for 7 days or control animals that were habituated to the tester, but not trained (Fig 2C). To determine APP protein expression in these neurons, tissue was dissected after learning, and cortices sectioned. Three animals were used per time point, and six sections were quantified per animal. Subjects that were trained showed an increase in APP protein levels compared to those that were not trained (Fig 2D). This expression was measured using ImageJ software, where AAVrg-positive cell bodies were identified as regions of interest (ROI). These ROIs were outlined and then 22C11 expression measured as integrated density. The corrected total cell fluorescence takes into account the background signal as well as area selected to generate a final fluorescence measure of APP 22C11. Using this methodology, APP protein was significantly increased in C8-projecing corticospinal neurons after 7 days of learning compared to control untrained animals (unpaired t-test). Collectively, this data suggests APP may play a role in motor learning.

DISCUSSION

Motor learning results in lasting anatomical changes where recurrent networks within the motor cortex involved in the task display an increase in connectivity. These same neurons show an increase in spine density and complexity, suggesting a selective reorganization of synaptic connections occurs within corticospinal neurons in response to motor learning. In efforts to understand what molecular determinants are responsible for this anatomical change, RNA sequencing revealed a distinct pattern of gene expression associated with learning. A cohort of γ-secretase substrates showed coordinate expression over time, where amyloid precursor protein (APP) was identified as a central "hub" gene with high correlation and connectivity in network modules identified through ingenuity pathway analysis. APP was identified as a central hub at all time points, as well as an upstream regulator where its AICD domains most likely play a role in transcriptional control.

To determine the direct role of APP in learning, we trained animals in forelimb reach task, and extracted protein from the motor cortex at 1 and 7 days after learning. Using antibodies against APP, we showed holo APP levels slightly increased with learning, where APP proteolytic fragments identified through a C-terminus antibody (Y188) showed a significant increase in response to learning. This suggests that APP protein is alternatively regulated in response to learning, although the direct cleavage product of APP still remains unclear. Further studies including mass spectroscopy will help identify the species of APP produced in response to learning and may shed light on which secretases are active during this learning paradigm.

To determine if cervical 8-projecting corticospinal neurons express APP protein and show changes in expression, a retrograde virus (AAVrg) was injected into the

spinal cord of mice to label CST neurons that specifically project to that spinal level engaged in the forelimb reach task. Animals that were trained in motor learning showed an increase in APP protein levels using the holo APP antibody, 22C11. This increase suggests APP protein is directly upregulated in CST neurons engaged in forelimb reach training.

Previous work has suggested that APP plays a role in synaptic biogenesis including synapse formation, strengthening and maturation [14]. Its role in long-term potentiation suggests APP plays a direct role in memory consolidation [17]. Most work focused on the role of APP in response to learning has been associated with hippocampal-mediated learning. Here for the first time we show that APP protein is a central hub in motor learning, and that it is alternatively spliced at 7 days after motor learning, a phase in which a new skill has been acquired and recurrent synaptic connections have been strengthened. The proteolytic fragments produced by APP learning remains unknown, and identifying their sequence might shed light on which secretases are active during learning, and the role APP may play during motor learning.

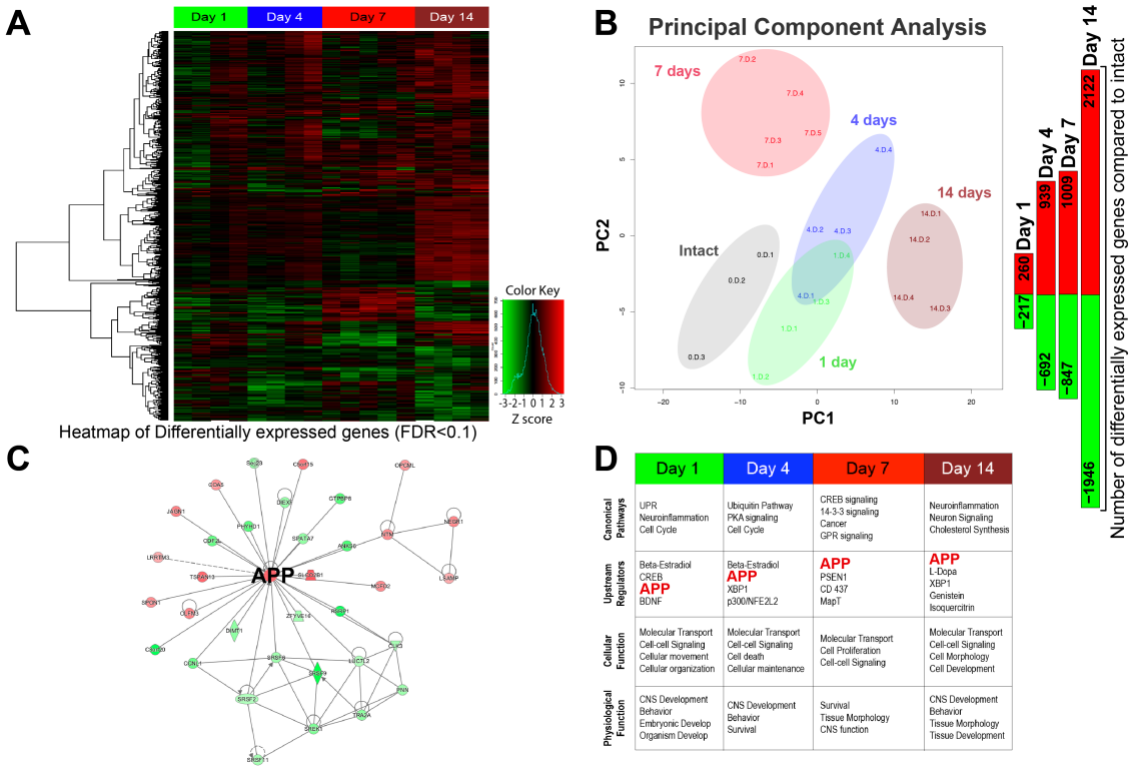

Figure 1. The Learning Transcriptome

A. A heat map of the learning transcriptome shows distinct patterns of gene expression in response to learning. APP showed a significant increase in mRNA expression in response to learning, along with 39 other γ-secretase substrates

B. PCA analysis shows these mRNA changes are driven along two eigen vectors (PC1 and PC2) as the animals undergo motor learning.

C. APP was identified as a hub gene using ingenuity pathway analysis (hub shown is at 14 days of learning).

D. Bioinformatics analysis further identified APP as an upstream regulator along all time points of learning.

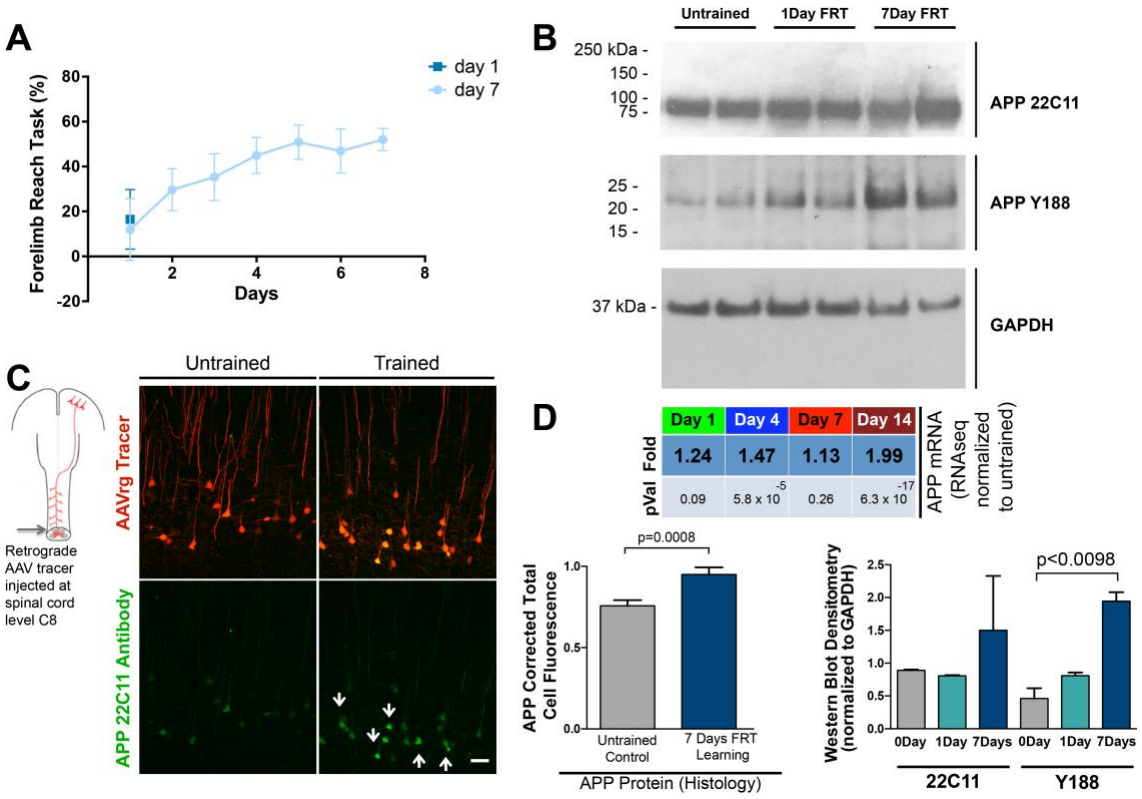

Figure 2. APP Protein levels in response to Learning.

A. Behavioral performance of mice as they learn forelimb reach task shows an acquisition of skill that can be quantitatively measured over time. Animals that were trained for 7 days in the task showed 50% accuracy, compared to those that were trained for 1 day only had 20% accuracy in the task.

B. Western blot analysis of these same subjects against APP protein shows a slight increase in holo APP protein levels. Using an antibody against the C-terminus of the protein revealed proteolytic fragments at 20 kDa that increases with motor learning.

C. Using AAVrg applied to C8 projecting corticospinal neurons shows APP localization in layer 5 neurons where trained subjects had more APP expression in these cells.

D. Quantification of western blots, histology, and mRNA sequencing shows APP protein upregulation in response to learning.

MATERIALS AND METHODS

Animals

All procedures involving animals were carried out in strict adherence to guidelines provided by The Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 2011), The Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH, 1986), The Animal Welfare Act/Regulations and subsequent amendments (PL 89-544), and The Veterans Health Administration Handbook 1200.07 "Use of Animals in Research" (2011); VA San Diego Healthcare System (VASDHS) Research Services Policy 01 section 151-04 (Institutional Animal Care and Use Committee, IACUC) and VASDHS IACUC Policy 03 (Pre and Post-procedural Care of Laboratory Rodents). The animal use protocol was approved by the VASDHS IACUC. A total of 20 C57BL/6 mice of both genders from Jackson Laboratories (Bar Harbor, ME) were obtained 6 – 8 weeks olds for the first behavioral experiments and the last experiment of 7 Cas9 background male and female mice were obtained at postnatal day 21. Mice were kept on a 12:12 light dark cycle and housed in groups of up to 5 mice per cage. They received standard enrichment nesting material and pieces of cardboard roll. Food restriction was implemented 5 days prior to training animals in the single pellet reaching test. Mice were fed 1.2 grams to 2.0 grams of standard rodent chow daily. Weights were monitored and food intake was adjusted to maintain a weight of > 85% body weight prior to food restriction.

Single Pellet Reaching Test

Young adult C57BL/6 (wildtype) mice were handled three weeks prior to shaping and training. Handling involved the acclimation of mice to trainer and trainer to mice with 15 minutes of leaving gloved hands in each cage followed up by regular lifting and labeling of tails in the first week of handling. The second week of handling involved the acclimation of mice to the enriched environment of a Plexiglas chamber with a wire mesh for the floor 0.5 cm from the floor so that missed rewards pellets could not be retrieved. The front of the chamber had an adjustable window in the center in which mice were able to reach out to a pedestal with an indentation for single 20 mg Noyes Precision reward pellets (sucrose pellets, Formula F, New Brunswick, NJ). Mice were allowed to explore the chamber with their cage mates for 20 minutes daily and then individually for 10 minutes. Sugar reward pellets were left at the front of the chamber during this week of handling. Mice were food restricted the last week of handling and two days prior to training, the mice were all shaped and conditioned to begin reaching for reward pellets. Mice were allowed to successfully reach and retrieve a pellet a maximum of two times in the first shaping day and a maximum of one time in the last shaping day. During this shaping procedure, preferred forelimb was identified.

Mice were then trained from as little as 5 to 14 consecutive days depending on the experiment. A successful reach was defined as extending a single forelimb through the reaching window such that the entire forepaw was outside the chamber walls. An unsuccessful hit or a miss was defined as the

extension of an entire forepaw outside of the chamber walls without retrieval of the sucrose reward pellet. Mice were initially allotted 30 trials to reach through the center aperture. After this initial experiment, mice were allotted 50 trials to reach through the center aperture in the experiments after. Forelimb reach success rate was measured by the total number of successful reaches divided by the total number of reaches. Each set of 50 reaches was recorded in bins of ten to help keep track of trials.

C8 spinal cord AAVrg Injections

Wild type C57BL/6 mice were heavily sedated and anesthetized with 1.5% oxygen and 4% isofluorane prior to the C8 spinal injection surgery. After initial knockdown of the animal, 1.5% oxygen with 1.5% isofluorane gas was used to keep the mice sedated in a mouse stereotax. A laminectomy was performed on C7 to cut the dura of the spinal cord and access the spinal cord with pulled pipette glass needles. Each mouse received 1ul of AAVrg virus injected into the spinal cord. Each lateral side of the spinal cord received one injection site at 0.3 mm lateral of the midline and 0.5 mm depth into the spinal cord. The pulled pipette glass needle was allowed to remain in the spinal cord after each injection for 60 seconds before removing it from the site. The mice were then sutured and given banamine, ampicillin, and ringers for the next three days. Mice were given one weeks to recover from the surgery and then the training protocol.

Immunohistochemical tissue processing

After they were trained, the mice were sacrificed; perfused intracardiacally with 4% paraformaldehyde, and the whole brain tissue was harvested. The whole brain was then sliced at coordinates 2mm anterior and 2 mm posterior to bregma and sectioned using a cryostat and microtome into 35 micron sections. Sections were then stained with APP 22C11 antibody (Ebioscience cat# 14-9749-80) at 1:100 dilution. Secondary antibody was applied at 1:1000 (Jackson mouse IgG 488). Images were taken with a confocal microscope.

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