Animal Use Protocol (AUP) Amendment Application

Use this form to request an amendment for your current Institutional Animal Care and Use Committee (IACUC) approved AUP. Complete items #1-3. Submit the AUP Amendment Application electronically to the Compliance Office (iacuc@ucr.edu), 207 UOB.

1. General Information:

AUP #:	A-20130033E
AUP Title:	1. EphB signaling in dendritic spine development 2. Targeting matrix metalloproteinases (MMPs) to treat dendritic spine malformation and behavioral defects in Fragile X Mice
Principal Investigator:	Iryna Ethell
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Submission Date:	11-05-2015

2. Type of amendment request (check all that apply):

X	Animal species/strain
X	Animal number
	Additional vivarium space (contact the Campus Veterinarian, 2-5845, if additional space will be needed)
	Animal source: animal transfer
X	Animal use procedure
	Animal care procedure
	Use of potentially hazardous substances ** (see below)
	Other (please specify in writing):

** Changes in the use of potentially hazardous substances must include an updated *Hazardous Materials Information* form (http://www.ora.ucr.edu/vet/Forms/AUP.doc section 8.0).

3. In narrative form briefly describe and justify the amendment request.

Be sure to include any related changes in:

- animal husbandry or housing (including environmental enrichment),
- anesthesia monitoring
- post-surgical monitoring

For changes in procedures, always address whether or not the consideration of adverse effects in the original protocol (section 5.0) thoroughly covers changes described in the amendment.

Species: Tg(Thy1-EGFP)MJrs mice

Funding Source: NIH

Objectives and Significance: In these mice pyramidal neurons in hippocampus and cerebral cortex are fluorescently labeled with GFP allowing for live imaging of dendritic spines, which are postsynaptic sites of majority excitatory synapses in the brain. The procedure described below will be used to monitor spine changes with during postnatal brain development in response to changes in MMP-9 activity and the expression of ephrin-B1 in astrocytes. Using the technique we will reduce the number of animals as we can monitor the changes in spines in the same mouse over a period of several days (Trachtenberg et al., 2002; Ligang et al.,

2014). We will also examine the effects of MMP-9 activation/inhibition on the organization of extracellular matrix and synapses by immunostaining of brain slices.

Experimental Procedures:

In vivo brain imaging:

Pre-surgery: Prior to surgery, all surgical instruments will be ultrasonically cleaned with detergent to remove any trace of blood and protein, then sterilized by immersion in 6% hydrogen peroxide, followed by 95% ethanol to remove caustic peroxide residue, then allowed to dry. Coverslips will be autoclaved and maintained prior to use in 70% ethanol. Mice will be anesthetized by IP injection of 0.5 ml (max per dose) of Ketamine/xylazine mix (80 mg/kg ketamine and 10 mg/kg xylazine). Adequate anesthesia will be assessed by paw pad pinch test, respiratory rhythm, righting reflex, and/or loss of corneal reflex. Respiratory rhythm, muscle tone, and reflex activity will continue to be monitored throughout the procedure in intervals of 10 minutes. Supplemental doses of ½ amount of initial dose will be provided as needed.

Surgery: Aseptic conditions will be observed for this survival surgery procedure. After anesthesia, animals scalps' will be shaved and cleaned with 3 alternating swipes of betadine and alcohol, opthalmic ointment will be administered to keep eyes moist through the procedure. A rectal temperature probe will be inserted into the animal and the temperature of the animal will be maintained with a homeothermic blanket system. Mice will be placed in a stereotactic frame. Skin will be infiltrated with lidocaine (2%) and opened over the burr hole site. Burr hole be made over A1 and the dura is carefully opened with a microneedle. The skull will be irrigated with ACDF during drilling to prevent the underlying brain from overheating. A small hole (~ 3mm in diameter), with location determined by coordinates with a stereotaxic atlas, will be drilled in the skull. Selected group of mice will be microinjected (see microinjection protocol below) prior to the window implant. We will insert a sterile 3 mm glass coverslip for spine imaging. Coverslip will be gently laid over the dura and glued to the skull with cyanoacrylate glue. Dental acrylic will be applied to the skull surface at the edges of the coverslip. Mice will be placed on a heating blanket during the procedure and observed for few hours prior returning to the cage. Return the animal to its routine housing only after it has recovered from anesthesia (i.e., the animal can maintain itself in sternal recumbency).

Post-surgery: The mice will be allowed to recover for several days during which time pain is treated with 0.3 ml (max per dose) of Buprenorphine by subcutaneous injection (s.c., an analgesic) given at 6-8 hour intervals for 48 hours following surgery, and as needed after that. After the implant procedure, animals will be monitored twice a day for signs of distress such as, vocalization, loss of appetite, prolonged inactivity, and ungroomed coats. Usually, with a combination of careful monitoring, post-operative antibiotics/analgesics, chronic implants are well managed in our preparations described above (see Lee et al., 2012). Animals will be inspected daily. Animals will be prematurely terminated if there is a visible sign of infection, coverslips cracks, wound margins bleed or animals display signs of withdraw or lethargy. If an animal exhibits signs of implant rejection/intolerance (e.g.

excessive scratching, pulling, infection), antibiotic/analgesics treatments (Baytril 2.5 SQ (0.5 ml max per dose) will be continued to treat infection of the scalp incision along with the usage of an Elizabethan collar as needed. If an animal continues to react negatively to the implant despite treatment, it will be euthanized and excluded from further study. At the end of the experiments, animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation.

Brain imaging: Animal will be imaged 7-14 days after the surgery. Mice will be anesthetized by IP injection of 0.5 ml (max per dose) of Ketamine/xylazine mix (80 mg/kg ketamine and 10 mg/kg xylazine) and placed in a stereotactic frame. Adequate anesthesia will be assessed by paw pad pinch test, respiratory rhythm, righting reflex, and/or loss of corneal reflex. Respiratory rhythm, muscle tone, and reflex activity will continue to be monitored throughout the procedure in intervals of 10 minutes. Imaging sessions will be 1-2 hours long. Supplemental doses of ½ amount of initial dose will be provided as needed.
Mice will be placed on a heating blanket during the procedure. The skull will be irrigated with ACDF during imaging to prevent from drying and overheating. At the end of the imaging experiment, animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation.

Microinjection protocol:
Pre-surgery: Prior to surgery, mice will be anesthetized by IP injection of 0.5 ml (max per dose) of Ketamine/xylazine mix (80 mg/kg ketamine and 10 mg/kg xylazine). Adequate anesthesia will be assessed by paw pad pinch test, respiratory rhythm, righting reflex, and/or loss of corneal reflex. Respiratory rhythm, muscle tone, and reflex activity will continue to be monitored throughout the procedure in intervals of 10 minutes. Supplemental doses of ½ amount of initial dose will be provided as needed.

Surgery: Aseptic conditions will be observed for this survival surgery procedure. After anesthesia, animals scalps' will be shaved and cleaned with 3 alternating swipes of betadine and alcohol, opthalmic ointment will be administered to keep eyes moist through the procedure. A rectal temperature probe will be inserted into the animal and the temperature of the animal will be maintained with a homeothermic blanket system. Mice will be placed in a stereotactic frame. Skin will be infiltrated with lidocaine (2%) and opened over the burr hole site. Burr hole be made over A1 and the dura is carefully opened with a microneedle. The skull will be irrigated with ACDF during drilling to prevent the underlying brain from overheating. A small hole (~ 1mm in diameter), with location determined by coordinates with a stereotaxic atlas, will be drilled in the skull over the injection site. Solution (usually about 0.5 microliters or 500 nl) will injected into the hippocampus or A1 by using a commercial oocyte-injection system (Drummond Scientific, Broomall, PA, USA) coupled to a glass micropipette (tip diameter about 100 micrometers). The injection system is mounted on a stereotaxic device that allows us to administer the injectate into the hippocampus or A1 cortex. The injection is carried out over a 5-min period to minimize cortical trauma, and the pipette is left in place for 15 min to prevent the solution from escaping through the injection site.
A single microinjection will be performed for each animal.
1. MMP inhibitor S3CT (250 μM) or recombinant-active MMP-9 (0.1 ug/ml, 2ul) will be performed (usually about 2 microliters perinjection) as previously described (Bozdagi O1,

Nagy V, Kwei KT, Huntley GW In vivo roles for matrix metalloproteinase-9 in mature hippocampal synaptic physiology and plasticity. J Neurophysiol. 2007 Jul;98(1):334-44. Epub 2007 May 9.) to WT, Fmr1 KO, MMP-9 KO or FMR1/MMP9 KO mice.
Following injection, the pipette will be carefully removed and the site closed. The skin will be closed with non-absorbable sutures. Mice will be placed on a heating blanket during the procedure and observed for few hours prior returning to the cage. Return the animal to its routine housing only after it has recovered from anesthesia (i.e., the animal can maintain itself in sternal recumbency). Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue analysis 6, 12 and 24 hours after the microinjection.
2. Microinjection of AAV2/5- gfaABC1D-eGFP; AAV7-gfaABC1D-ephrin-B1.SV40, or AAV7-gfaABC1D-tdTomato.SV40 will be performed (~ 2 microliters per injection) to the cortex or hippocampus of WT or EphB KO mice. Following injection, the pipette will be carefully removed. In one group of animals the site closed. The skin will be closed with non-absorbable sutures. The sutures will later be removed (7-14 d after the surgery) under isoflurane anesthesia. Mice will be placed on a heating blanket during the procedure and observed for few hours prior returning to the cage. Return the animal to its routine housing only after it has recovered from anesthesia (i.e., the animal can maintain itself in sternal recumbency). In a second group of animals window will be implanted immediately after the microinjection (see in vivo imaging for details).

Post-surgery: The mice will be allowed to recover for several days during which time pain is treated with 0.3 ml (max per dose) of Buprenorphine by subcutaneous injection (s.c., an analgesic) given at 6-8 hour intervals for 48 hours following surgery, and as needed after that. After the implant procedure, animals will be monitored twice a day for signs of distress such as, vocalization, loss of appetite, prolonged inactivity, and ungroomed coats. Usually, with a combination of careful monitoring, post-operative antibiotics/analgesics, chronic implants are well managed in our preparations described above (see Lee DJ, Hsu MS, Seldin MM, Arellano JL, Binder DK., Exp Neurol. 2012 May;235(1):246-55). If an animal exhibits signs of implant rejection/intolerance (e.g. excessive scratching, pulling, infection), antibiotic/analgesics treatments (Baytril 2.5 SQ (0.5 ml max per dose) will be continued to treat infection of the scalp incision along with the usage of an Elizabethan collar as needed. If an animal continues to react negatively to the implant despite treatment, it will be euthanized and excluded from further study. At the end of the experiments, animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation.

Intraventricular cannulation protocol:
Pre-surgery: Prior to surgery, mice will be anesthetized by IP injection of 0.5 ml (max per dose) of Ketamine/xylazine mix (80 mg/kg ketamine and 10 mg/kg xylazine). Adequate anesthesia will be assessed by paw pad pinch test, respiratory rhythm, righting reflex, and/or loss of corneal reflex. Respiratory rhythm, muscle tone, and reflex activity will continue to be monitored throughout the procedure in intervals of 10 minutes. Supplemental doses of ½ amount of initial dose will be provided as needed.

Surgery: Aseptic conditions will be observed for this survival surgery procedure. After anesthesia, animals scalps' will be shaved and cleaned with 3 alternating swipes of

betadine and alcohol, opthalmic ointment will be administered to keep eyes moist through the procedure. A rectal temperature probe will be inserted into the animal and the temperature of the animal will be maintained with a homeothermic blanket system. Mice will be placed in a stereotactic frame. Skin will be infiltrated with lidocaine (2%) and opened over the burr hole site. Burr hole will be made over right lateral ventricle, with location determined by coordinates with a stereotaxic atlas, and the dura is carefully opened with a microneedle. The skull will be irrigated with ACDF during drilling to prevent the underlying brain from overheating. A small hole with location determined by coordinates with a stereotaxic atlas, will be drilled in the skull. Mice weighing 25-30g will be stereotaxically implanted with either one or two 26-gauge stainless steel guide cannulae targeted at the right lateral ventricle. An osmotic minipump (Alzet model 2002; flow rate, 0.5 ml /hr; Alza Corporation), aseptically prefilled with either saline, control 0.6% dimethylsulfoxide (DMSO), or 2.5 □M SB3CT in saline containing 0.6% DMSO attached to a cannula via polyethylene tubing, and prewarmed in 0.9% NaCl at 37°C for 4 hr, will be placed subcutaneously in the nuchal area. The cannula will be implanted stereotaxically with the tip in the right lateral ventricle (20.8 mm anteroposterior; 11.5 mm lateral; and 23.6 mm dorsal) (Paxinos and Watson, 1982). Cannula will be secured firmly to the skull with dental cement and 3-4 anchor screws. In several preliminary experiments, acute injection of Evans blue dye and postmortem histological examination were performed to verify cannula patency and cannula. The skin will be closed with non-absorbable sutures. The sutures will later be removed (7-14 d after the surgery) under isoflurane anesthesia. Mice will be placed on a heating blanket during the procedure and observed for few hours prior returning to the cage. Prior to anesthetic recovery pain is treated with 0.3 ml (max per dose) of Buprenorphine by subcutaneous injection (s.c., an analgesic). Return the animal to its routine housing only after it has recovered from anesthesia (i.e., the animal can maintain itself in sternal recumbency).

Post-surgery: The mice will be allowed to recover for several days during which time pain is treated with 0.3 ml (max per dose) of Buprenorphine by subcutaneous injection (s.c., an analgesic) given at 6-8 hour intervals for 48 hours following surgery, and as needed after that. Animals will be allowed to recover for 4 d after surgery before treatment with active MMP-9 or MMP-9 inhibito. MMP inhibitor S3CT (250 μM) or recombinant-active MMP-9 (0.1 ug/ml, 2ul) will be delivered via an infusion cannula connected to a Hamilton syringe driven by a syringe pump (200 nl/min; 2 µl total volume) as previously described (Bozdagi O1, Nagy V, Kwei KT, Huntley GW In vivo roles for matrix metalloproteinase-9 in mature hippocampal synaptic physiology and plasticity. J Neurophysiol. 2007 Jul;98(1):334-44. Epub 2007 May 9). The solutions will be infused once per day for 7 days. Animals will be monitored twice a day for signs of distress such as, vocalization, loss of appetite, prolonged inactivity, and ungroomed coats. Usually, with a combination of careful monitoring, post-operative antibiotics/analgesics, chronic implants are well managed in our preparations described above (see Lee DJ, Hsu MS, Seldin MM, Arellano JL, Binder DK., Exp Neurol. 2012 May;235(1):246-55). If an animal exhibits signs of implant rejection/intolerance (e.g. excessive scratching, pulling, infection), antibiotic/analgesics treatments (Baytril 2.5 SQ (0.5 ml max per dose) will be continued to treat infection of the scalp incision along with the usage of an Elizabethan collar as needed. If an animal continues to react negatively to the implant despite treatment, it will be euthanized and excluded from further

study. At the end of the experiments, animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation.

Handling and Side effects: During procedure adequate anesthesia will be assessed by paw pad pinch test, respiratory rhythm, righting reflex, and/or loss of corneal reflex. Respiratory rhythm, muscle tone, and reflex activity will continue to be monitored throughout the procedure in intervals of 10 minutes. Supplemental doses of ½ amount of initial dose will be provided as needed. Animals will be monitored every 5 minutes for respiratory rate and resumption of ambulatory activity while resting on a homeothermic heating blanket. Once the animals are ambulatory, they are moved to a holding cage.

After the procedure, animals will be monitored twice a day for signs of distress such as, vocalization, loss of appetite, prolonged inactivity, and un-groomed coats. The mice will be allowed to recover for several days during which time pain is treated with Buprenorphine (s.c., an analgesic). Usually, with a combination of careful monitoring, post-operative antibiotics/analgesics. If an animal exhibits signs of implant rejection/intolerance (e.g. excessive scratching, pulling, infection), antibiotic/analgesics treatments will be continued along with the usage of an Elizabethan collar as needed. If an animal continues to react negatively to the implant despite treatment, it will be euthanized and excluded from further study. Animals will be monitored for signs of distress, such as vigorous scratching of wound site, vocalization, loss of appetite, prolonged inactivity, and ungroomed coats. If animals display multiple signs of distress the Campus Veterinarian will be consulted and the recommended treatment followed, or the animal euthanized. Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.).

Number of animals: We will need 40-60 mice in each group (see 6 groups below) over a period of three years. Total: 300 mice or 100 mice per year.

Group	mice	Procedure 1	Procedure 2	Procedure 3	Procedure 3
1. Acute effects of MMP9 activation or inhibition on synapses	3 time points x 3 treatments x 5 mice=45	Microinjection of control saline, MMP inhibitor S3CT or recombinant-active MMP-9	Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue		

			analysis 6, 12 or 24 h post injection		
2. Chronic effects of MMP9 activation or inhibition on synapses	4 genotypes x 3 treatments x 5 mice=60	Intraventricular cannulation	Control saline, MMP inhibitor S3CT or recombinant-active MMP-9 will be delivered via an infusion cannula (7-14 days after implanting cannula). The solutions will be infused once per day for 7 days.	Animals will be euthanized (2-4 weeks after microinjection) by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue analysis	
3. Affects of ephrinB1 overexpression in astrocytes on synapses	4 genotypes (WT, Thy1GFP, Thy1GFP/ EphB1,2,3 KO) x 10 mice = 40 mice	Microinjection of AAV2/5-gfaABC1D-eGFP; AAV7-gfaABC1D-ephrin-B1.SV40, or AAV7-gfaABC1D-tdTomato.SV40	Implantation of window in the skull (same day as microinjection)	In vivo imaging (2-4 weeks after the injection)	Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue analysis immediately after the imaging
4. Effects of ephrinB1 overexpression in	4 genotypes (WT, Thy1GFP,	Microinjection of AAV2/5-gfaABC1D-eGFP; AAV7-	Animals will be euthanized (2-4 weeks after microinjection)		

astrocytes on synapses	Thy1GFP/ EphB1,2,3 KO) x 10 mice = 40 mice	gfaABC1D-ephrin-B1.SV40, or AAV7-gfaABC1D-tdTomato.SV40	by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue analysis		
5. Effects of ephrinB1 deletion in astrocytes on synapses	4 genotypes (Floxed ephrinB1/ GFAP-ERT2Cre/tdTomato/Thy1GFP, floxed ephrinB1, Floxed/tdTomato/Thy1GFP, ephrinB1/ GFAP-Cre, GRAP-Cre/tdTomato/Thy1GFP) x 10 mice = 40 mice	Implant glass window in the skull	In vivo imaging (2-4 weeks after the injection)	Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed by decapitation for tissue analysis	
6. Effects of Fmr1 deletion in neurons or astrocytes on synapses	4 genotypes (Floxed Fmr1/GFAP-ERT2Cre/tdTomato/Thy1GFP,	Implant glass window in the skull	In vivo imaging (2-4 weeks after the window implant)	Animals will be euthanized by a single injection of sodium pentobarbital (100 mg/kg, i.p.) followed	

	floxed Fmr1/Thy1 GFP, Floxed ephrinB1/ CamKII-Cre/Thy1GFP/tdTomato, Floxed Fmr1/Nex-Cre/Thy1GFP/tdTomato) x 10 mice = 40 mice			by decapitation for tissue analysis	

Pain and Distress Category 3: Moderate to severe distress. Animals will potentially experience more than momentary or slight pain or distress that will be alleviated through the use of anesthetics and/or analgesics.

Personnel and Training. The procedures will be performed by Jordan Koeppen, Sonia Afroz and Amanda Nguyen, who will be trained on in vivo procedure by Dr. Erica Arroyo in the lab of Carlos Portera at the University of California Los Angeles and Dr. Binder's lab at UCR on microinjection and cannulation procedures. The training will involve three 5 h - long training sessions. Dr. Arroyo is very experienced as she performs in vivo imaging procedure weekly. Once my laboratory obtains approval for the procedures, Jordan Koeppen, Sonia Afroz and Amanda Nguyen will start performing the procedures. In vivo imaging requires advanced technical skills. Prior training on live animals, training will involve extensive learning of procedure without animal use or using animal corpses used for primary cultures and brain slices.

References:

Trachtenberg JT, Chen BE, Knott GW, Feng F, Sanes JR, Welker E, Svoboda K. 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex Nature 420, 788-794.

Ligang Gu, Stefanie Kleiber, Lena Schmid, Felix Nebeling, Miriam Chamoun, Julia Steffen, Jens Wagner, and Martin Fuhrmann. Long-Term In Vivo Imaging of Dendritic Spines in the Hippocampus Reveals Structural Plasticity. The Journal of Neuroscience, 15 October 2014, 34(42):13948-13953.

Lee DJ, Hsu MS, Seldin MM, Arellano JL, Binder DK. 2012. Decreased expression of the glial water channel aquaporin-4 in the intrahippocampal kainic acid model of epileptogenesis. Exp Neurol. 235(1):246-255.

Bozdagi O1, Nagy V, Kwei KT, Huntley GW In vivo roles for matrix metalloproteinase-9 in mature hippocampal synaptic physiology and plasticity. J Neurophysiol. 2007 Jul;98(1):334-44. Epub 2007 May 9

Committee Use Only

Final Disposition of this protocol:

_____ Approved by Chair/Vice-Chair

___XX_____ Approved by IACUC Review

_____ Not Approved by IACUC Review

_____ Withdrawn by Investigator

Date of Action: __3__/__22____/__16____