<u>Specific Aim 1</u>: Determine if EVs from human subjects with PAH induce pulmonary hypertensive changes in healthy mice (figure 1)

Species/Strain/Gender: Mice, C57BL/6, male
Use: Infuse with EVs isolated from human subjects with PAH, control subjects
Analysis: Measurement of right ventricular (RV) peak systolic pressure, histologic measurements
Number animals needed:
8 mice, PAH subject EVs
8 mice, control subject EVs
8 mice, vehicle (PBS)
total of 24 animals per experiment
Total number of experiments: 2
Total number of animals for Aim 1: 48

Summary of methods: Male C57BL/6 mice will arrive in the animal facility. Seven days later, cohorts will receive IV injections, via tail vein, of 100ug of PAH subject EVs, 100ug of control subject EVs or an equal volume (100ul) of vehicle (PBS). 28 days later, mice will be anesthetized and RV peak systolic pressures will be measured. Mice will then be euthanized and lungs and hearts will be collected for histological analysis (figure 2).

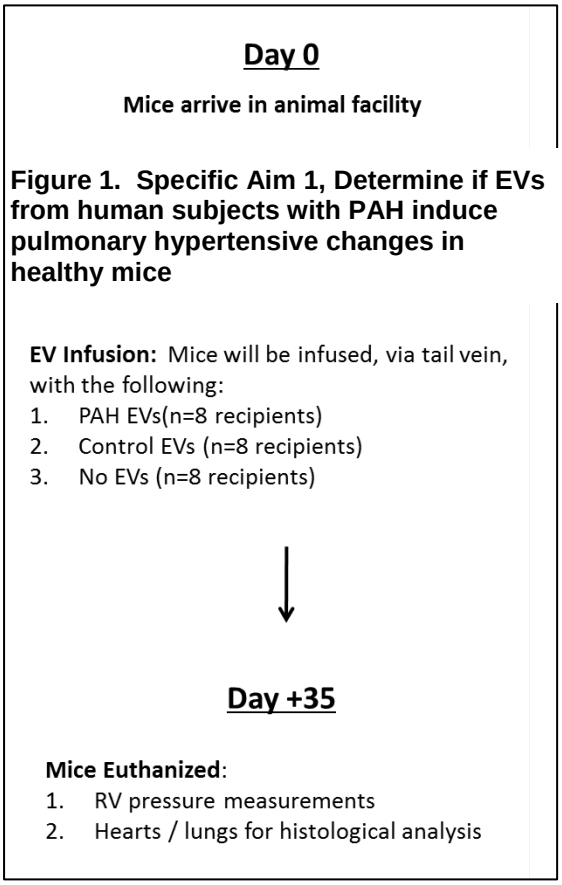

Figure 1. Specific Aim 1, Determine if EVs from human subjects with PAH induce pulmonary hypertensive changes in healthy mice

<u>Specific Aim 2</u>: Determine if bone marrow cells cultured with EVs from human subjects with PAH induce pulmonary hypertensive changes when transplanted into healthy mice (figure 2)

Species/Strain/Gender: Mice, C57BL/6, male
Use: Infuse with whole bone marrow cells (WBM) cultured with EVs isolated from human subjects with PAH, control subjects into irradiated mice.
Analysis: Measurement of right ventricular (RV) peak systolic pressure, histologic measurements
Number animals needed:
2 mice, WBM donors
8 mice, WBM + PAH subject EVs
8 mice, WBM + control subject EVs
8 mice, WBM + vehicle (PBS)
8 mice, no WBM, EVs, radiation
total of 34 animals per experiment
Total number of experiments: 2
Total number of animals for Aim 2: 68

Summary of methods: Male C57BL/6 mice will arrive in the animal facility. Seven days later, two mice will be euthanized and their WBM isolated and cultured with 100ug of PAH subject EVs, 100ug of control subject EVs or an equal volume (100ul) of vehicle (PBS) vehicle. Two days later, cohorts of mice will receive 950 cGy of total body irradiation and will be infused, via tail vein, with WBM cultured with EVs or vehicle. A control cohort will not be irradiated or infused with EVs or WBM. 28 days later, mice will be anesthetized and RV peak systolic pressures will be measured. Mice will then be euthanized and lungs and hearts will be collected for histological analysis (figure 2).

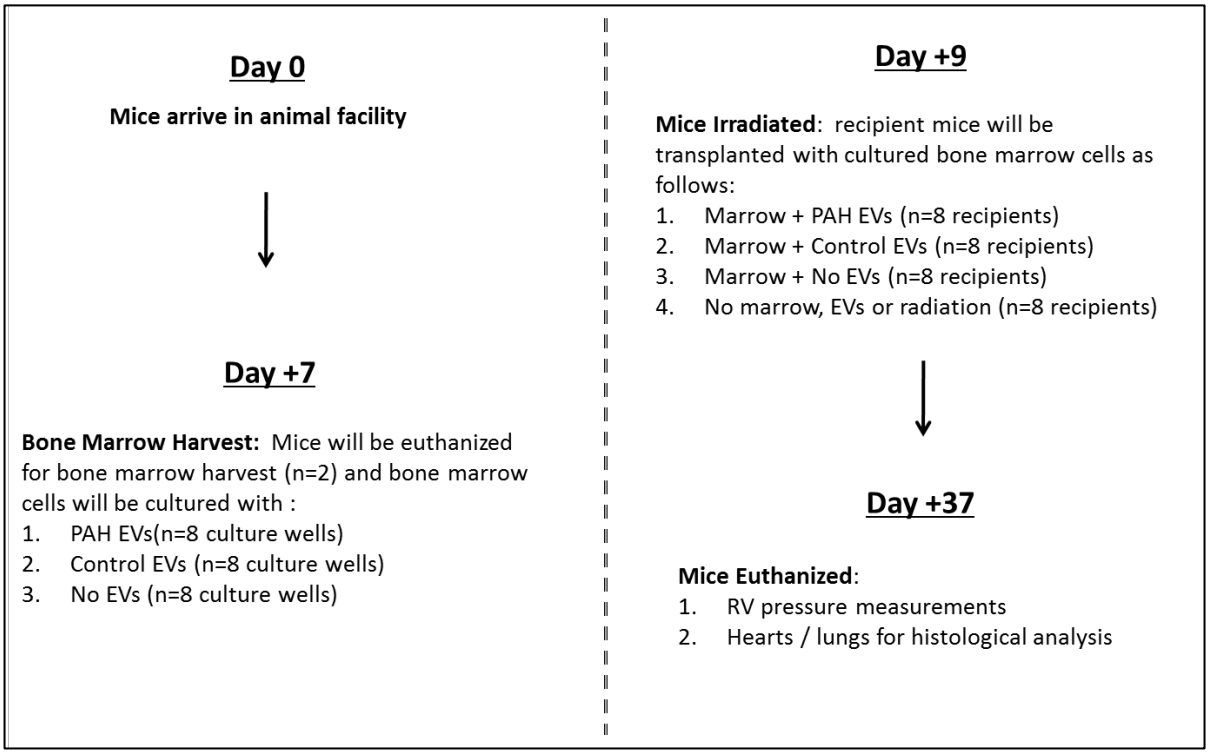

Figure 2. Specific Aim 2, Determine if bone marrow cells cultured with EVs from human subjects with PAH induce pulmonary hypertensive changes when transplanted into healthy mice

1) Generate chronic + binge ethanol exposure models:

1. Male and female rats will arrive and be adapted to animal care facility for 1-2 weeks
2. Experimental Weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content for 8 weeks.
3. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.
4. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8.
5. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

1-TIME LINE FOR ETHANOL EXPOSURE MODEL

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation
Wks 3-10 (Exp Wk1-8)	Isocaloric liquid diets	Chronic ethanol feeding model
Wks 9-10 (Exp Wk 7-8)	i.p. or gavage ethanol, 2-3 g/kg Mon, Wed, Fri afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2 inhalation	Harvest brains for study

2) Ethanol plus i.p. low-dose nitrosamine (NNK, NDEA, STZ) exposure models.

1. Male and female rats will arrive and be adapted to animal care facility for 1-2 weeks
2. Experimental weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content for 8 weeks.
3. Experimental weeks 1-8, co-administer subsets in each diet group, i.p. NNK (1-2 mg/kg) or saline, 3 times per week (MWF). Alternatively, NDEA (2-3 mg/kg, MWF) or STZ (25 mg/kg MWF Exp Wks 1 and 2 only) could be substituted for NNK.
4. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol fed rats on Mon, Wed, Fri afternoons. i.p. saline treat controls.
5. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8

6. End of experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide. Harvest brains and other organs and tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

2-TIME LINE FOR ETHANOL AND i.p. NNK EXPOSURES

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation
Wks 3-10 (Exp Wk1-8)	Isocaloric control or ethanol-containing liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk1-8)	NNK exposures-1-2 mg/kg i.p. 3x/wk-MWF); alternatively, NDEA (2-3 mg/kg, 3x/wk-MWF) or STZ (25 mg/kg, MWF Exp Wks 1-2 only) could be substituted for NNK.	Generate ethanol ± NNK models; alternatively, ethanol + NDEA or STZ
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study

3) Ethanol and i.c. nitrosamine (NNK, STZ) exposure models.

1. Male and female rats will arrive and be adapted to animal care facility for 1-2 weeks
2. Experimental Day 1, we will anesthetize with ketamine/xylazine. Shave and prep head for stereotaxic injection. Position rat in stereotaxic frame and identify coordinates relative to Bregma. Lubricate eyes. Make incision and burr hole. Lidocaine anesthetize peri-incision site. Deliver a single i.c. injection (1-3 µl) of STZ (50 mg/kg). Alternatively use NNK-2 mg/kg instead of STZ. Seal burr hole with sterile bone wax and suture incision. Monitor every 10 minutes for 2 h then at 30 min intervals until completely recovered and moving freely.
3. Experimental Days 1-3, allow for continued recovery from minor surgery and anesthesia including treatment with buprenorphine (0.01-0.05 mg/kg) twice/day as needed for pain and distress.
4. Experimental Day 3 through Week 8, maintain rats on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content.
5. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.
6. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8
7. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

3-TIME LINE FOR ETHANOL AND i.c. STZ OR NNK EXPOSURES

Time	Treatment	Goal
Weeks 1 and 2	Housing in	Environmental Adaptation
Week3, Exp Day 1	Stereotaxic i.c. administration of STZ or NNK under ketamine/xylazine anesthesia	Generate i.c. STZ or NNK models of brain insulin resistance
Week 3, Exp Day 1-3	Recovery from minor surgery and buprenorphine treatment as needed	Complete recovery before ethanol feeding
Weeks 3-10; Exp	Maintain on Isocaloric control or	Chronic ethanol feeding model

Day 4-wk 8	ethanol-containing liquid diets	
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study

4) Ethanol and smoking exposure models (Separate protocol to be submitted to th

1. Male and female rats will arrive at the Rodent Facility and be adapted for 1-2 weeks
2. Experimental Weeks 1-8, 6-hour daily exposures to direct and side-stream cigarette smoke in specialized smoking chamber. Control exposures to room air under same chamber conditions.
3. Experimental Weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content.
4. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.
5. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.
6. Experimental Week 8, Transfer tissue samples from to

4-TIME LINE FOR ETHANOL AND TOBACCO SMOKE EXPOSURES

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation
Wks 3-10 (Exp Wk1-8)	Isocaloric control or ethanol-containing liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk1-8)	Tobacco Smoke or room air exposures in controlled chambers; 6 h/day	Generate ethanol ± NNK models
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study
Wk 10 (Exp Wk 8)	Transfer tissue to for storage and study	Tissue banking

5) Generate chronic + binge ethanol exposure with rescue models:

1. Male and female rats will arrive and be adapted to animal care facility for 1-2 weeks
2. Experimental Weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content for 8 weeks.
3. Beginning from Experimental Week 1, 3, 5, or 7 and continuing through Experimental Week 8, treat rats daily with: 1) anti-diabetes drugs (PPAR agonists including either a hybrid PPAR-delta/gamma agonist (T3D959; i.p. or gavage), or a PPAR-delta (L-165,041) plus PPAR-gamma (Fmoc-Leu) agonist by i.p. injection; 2) myriocin-ceramide inhibitor (gavage); 3) N-acetylcysteine or superoxide dismutase antioxidant (gavage); 4) PPAR agonist plus myriocin; 5) PPAR agonist + anti-oxidant; 6) myriocin + anti-oxidant; or 7) PPAR agonist + myriocin + anti-oxidant. Water gavaged or saline i.p. treated controls will be studied in parallel.
4. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.

5. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8
6. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

5-TIME LINE FOR ETHANOL EXPOSURE MODEL PLUS RESCUE THERAPY

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation
Wks 3-10 (Exp Wk1-8)	Isocaloric liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk 1-8)	Treat with PPAR agonists, anti-ceramide, anti-oxidant, PPAR agonists + anti-ceramide, PPAR agonist + anti-oxidant; anti-ceramide+ anti-oxidant; PPAR agonist+ anti-ceramide + anti-oxidant beginning Exp Wks 1, 3, 4, or 7 and continue through Exp Wk 8	Assess time course effects of PPAR agonists, anti-ceramide, and anti-oxidant mono, duplex, and triple therapy for reducing or preventing cognitive impairment and neurodegeneration
Wks 9-10 (Exp Wk 7-8)	i.p. or gavage ethanol, 2-3 g/kg Mon, Wed, Fri afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2 inhalation	Harvest brains for study

6) Ethanol plus i.p. low-dose nitrosamine (NNK, NDEA, STZ) exposure with rescue models.

1. Male and female rats will arrive and be adapted to animal care facility for 1-2 weeks
2. Experimental weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content for 8 weeks.
3. Experimental weeks 1-8, co-administer subsets in each diet group, i.p. NNK (1-2 mg/kg) or saline, 3 times per week (MWF). Alternatively, NDEA (2-3 mg/kg, MWF) or STZ (25 mg/kg MWF Exp Wks 1 and 2 only) could be substituted for NNK..
4. Beginning from Experimental Week 1, 3, 5, or 7 and continuing through Experimental Week 8, treat rats daily with: 1) anti-diabetes drugs (PPAR agonists including either a hybrid PPAR-delta/gamma agonist (T3D959; i.p. or gavage), or a PPAR-delta (L-165,041) plus PPAR-gamma (Fmoc-Leu) agonist by i.p. injection; 2) myriocin-ceramide inhibitor (gavage); 3) N-acetylcysteine or superoxide dismutase antioxidant (gavage); 4) PPAR agonist plus myriocin; 5) PPAR agonist + anti-oxidant; 6) myriocin + anti-oxidant; or 7) PPAR agonist + myriocin + anti-oxidant. Water gavaged or saline i.p. treated controls will be studied in parallel.
5. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol fed rats on Mon, Wed, Fri afternoons. i.p. saline treat controls.
6. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8
7. End of experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide. Harvest brains and other organs and tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

6-TIME LINE FOR ETHANOL AND i.p. NNK EXPOSURES PLUS RESCUE THERAPY

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation

Wks 3-10 (Exp Wk1-8)	Isocaloric control or ethanol-containing liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk1-8)	NNK exposures-1-2 mg/kg i.p. 3x/wk-MWF); alternatively, NDEA (2-3 mg/kg, 3x/wk-MWF) or STZ (25 mg/kg, MWF Exp Wks 1-2 only) could be substituted for NNK.	Generate ethanol ± NNK models; alternatively, ethanol + NDEA or STZ
Wks 3-10 (Exp Wk 1-8)	Treat with PPAR agonists, anti-ceramide, anti-oxidant, PPAR agonists + anti-ceramide, PPAR agonist + anti-oxidant; anti-ceramide+ anti-oxidant; PPAR agonist+ anti-ceramide + anti-oxidant beginning Exp Wks 1, 3, 4, or 7 and continue through Exp Wk 8	Assess time course effects of PPAR agonists, anti-ceramide, and anti-oxidant mono, duplex, and triple therapy for reducing or preventing cognitive impairment and neurodegeneration
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study

7) Ethanol and i.c. nitrosamine (NNK, STZ) exposure with rescue models.

1. Male and female rats will arrive and be adapted to [redacted] animal care facility for 1-2 weeks
2. Experimental Day 1, administer a single i.c. injection of STZ (or NNK) under ketamine/xylazine anesthesia and using a stereotaxic frame
3. Experimental Days 1-3, allow recovery from minor surgery and anesthesia including treatment with buprenorphine as needed.
4. Experimental Day 3 through Week 8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content.
5. Beginning from Experimental Week 1, 3, 5, or 7 and continuing through Experimental Week 8, treat rats daily with: 1) anti-diabetes drugs (PPAR agonists including either a hybrid PPAR-delta/gamma agonist (T3D959; i.p. or gavage), or a PPAR-delta (L-165,041) plus PPAR-gamma (Fmoc-Leu) agonist by i.p. injection; 2) myriocin-ceramide inhibitor (gavage); 3) N-acetylcysteine or superoxide dismutase antioxidant (gavage); 4) PPAR agonist plus myriocin; 5) PPAR agonist + anti-oxidant; 6) myriocin + anti-oxidant; or 7) PPAR agonist + myriocin + anti-oxidant. Water gavaged or saline i.p. treated controls will be studied in parallel.
6. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.
7. Experimental weeks 7 and 8, perform neurobehavioral Morris water maze tests in mornings; Tuesday-Friday of Exp Wk 7 and Friday of Exp Wk 8
8. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

7-TIME LINE FOR ETHANOL AND i.c. STZ OR NNK EXPOSURES PLUS RESCUE THERAPY

Time	Treatment	Goal
Weeks 1 and 2	Housing in [redacted]	Environmental Adaptation
Week3, Exp Day 1	Stereotaxic i.c. administration of STZ or NNK under ketamine/xylazine anesthesia	Generate i.c. STZ or NNK models of brain insulin resistance

Week 3, Exp Day 1-3	Recovery from minor surgery and buprenorphine treatment as needed	Complete recovery before ethanol feeding
Weeks 3-10; Exp Day 4-wk 8	Maintain on Isocaloric control or ethanol-containing liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk 1-8)	Treat with PPAR agonists, anti-ceramide, anti-oxidant, PPAR agonists + anti-ceramide, PPAR agonist + anti-oxidant; anti-ceramide+ anti-oxidant; PPAR agonist+ anti-ceramide + anti-oxidant beginning Exp Wks 1, 3, 4, or 7 and continue through Exp Wk 8	Assess time course effects of PPAR agonists, anti-ceramide, and anti-oxidant mono, duplex, and triple therapy for reducing or preventing cognitive impairment and neurodegeneration
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wks 9-10 (Exp Wk 7-8)	Morris Water Maze tests in AM; Tu-Fri Exp Wk 7 and Fri Exp Wk 8	Assess spatial learning & memory
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study

8) Ethanol and smoking exposure with rescue models.

1. Male and female rats will arrive at the Rodent Facility and be adapted for 1-2 weeks
2. Experimental Weeks 1-8, 6-hour daily exposures to direct and side-stream cigarette smoke in specialized smoking chamber. Control exposures to room air under same chamber conditions.
3. Experimental Weeks 1-8, maintain on isocaloric liquid diets containing 0%. 8%, 18%, 24%, or 36% ethanol by caloric content.
4. Beginning from Experimental Week 1, 3, 5, or 7 and continuing through Experimental Week 8, treat rats daily with: 1) anti-diabetes drugs (PPAR agonists including either a hybrid PPAR-delta/gamma agonist (T3D959; i.p. or gavage), or a PPAR-delta (L-165,041) plus PPAR-gamma (Fmoc-Leu) agonist by i.p. injection; 2) myriocin-ceramide inhibitor (gavage); 3) N-acetylcysteine or superoxide dismutase antioxidant (gavage); 4) PPAR agonist plus myriocin; 5) PPAR agonist + anti-oxidant; 6) myriocin + anti-oxidant; or 7) PPAR agonist + myriocin + anti-oxidant. Water gavaged or saline i.p. treated controls will be studied in parallel.
5. Experimental weeks 7 and 8, binge ethanol expose (2-3 g/kg; i.p. or gavage) chronic ethanol diet fed rats on Mon, Wed, Fri afternoons. Saline treat controls.
6. End of Experimental Week 8, sacrifice rats by inhalation of isoflurane or carbon dioxide Harvest brains and other tissues. Rats may also be sacrificed at earlier intervals to monitor molecular and structural changes of neurodegeneration.

8-TIME LINE FOR ETHANOL AND TOBACCO SMOKE EXPOSURES PLUS RESCUE THERAPY

Time	Treatment	Goal
Wks 1 and 2	Housing in	Environmental Adaptation
Wks 3-10 (Exp Wk1-8)	Isocaloric control or ethanol-containing liquid diets	Chronic ethanol feeding model
Wks 3-10 (Exp Wk1-8)	Tobacco Smoke or room air exposures in controlled chambers; 6 h/day	Generate ethanol ± NNK models
Wks 3-10 (Exp Wk 1-8)	Treat with PPAR agonists, anti-ceramide, anti-oxidant, PPAR agonists + anti-ceramide, PPAR	Assess time course effects of PPAR agonists, anti-ceramide, and anti-oxidant mono, duplex,

	agonist + anti-oxidant; anti-ceramide+ anti-oxidant; PPAR agonist+ anti-ceramide + anti-oxidant beginning Exp Wks 1, 3, 4, or 7 and continue through Exp Wk 8	and triple therapy for reducing or preventing cognitive impairment and neurodegeneration
Wks 9-10 (Exp Wk 7-8)	Binge i.p. or gavage ethanol, 2-3 g/kg or water M W F afternoons	Generate chronic + binge ethanol exposure models
Wk 10 (Exp Wk 8)	Sacrifice with isoflurane or CO_2	Harvest brains for study
Wk 10 (Exp Wk 8)	Transfer tissue to for storage and study	Tissue banking

Please include a diagram or timeline to explain experiments and time points. The timeline should begin with animal arrival in the facility and/or the first procedure and end with euthanasia. Note when individual animals will be used for more than one procedure. **Please see below.**