SIGNIFICANCE

Our goal is to develop the first effective drug to treat traumatic brain injury (TBI), which ranges from mild concussion to serious injury and often results in chronic neurological and cognitive deficits. TBI most commonly results from falls, violence, sports-related impacts, vehicle accidents, and explosions, and in addition to being the leading cause of death and disability in children and young adults, it is a major health risk for combat military personnel and the elderly. Moderate-to-severe injuries can have a lasting impact on quality of life by impairing important functions, such as attention, decision making, reasoning, learning, memory, movement, vision, and hearing, and some patients develop additional long-term medical complications such as Alzheimer's disease.[1] Other consequences may include chronic pain, emotional liability, personality changes, anxiety, depression, and epilepsy. In North America alone, more than 1.7 M individuals are treated annually for TBI at an estimated cost >$75 B; of these about 52,000 die and 275,000 are hospitalized.[2] Furthermore, more than 5 million TBI victims in the US require permanent help in performing daily activities. The global scope of this problem is even worse, as the WHO places the annual global incidence for TBI at 10 million people.

There is a 'golden period' of potential opportunity after TBI for therapeutic suppression of pathological biochemical cascades leading to secondary neuronal damage, thereby improving functional outcomes after injury. However, no drug for this application exists, and the standard of care for acute treatment of moderate-to-severe TBI is limited to "off-label" use of existing drugs that merely provide transient relief of symptoms without addressing the underlying neurodegeneration that drives the process. Despite numerous phase III clinical trials, no new drug has been approved for more than 25 years.[3] *There is thus an urgent unmet need for a new drug that inhibits the progression of nerve damage and prevents or attenuates the cognitive impairment associated with serious brain injury.* Notably, the commercial value of such a drug is estimated to be >$3 B annually.[4] The discovery of a neuroprotective compound that mitigates the varied pathological effects of TBI by suppressing secondary neuronal damage following head trauma is the "holy grail" for therapeutic intervention. Accordingly, the proposed research is directed toward studying a promising new approach to treat TBI. *Our research is significant because: (1) we will validate that the sigma 2 receptor (Sig2R), which has not previously been associated with TBI, can be modulated to reduce secondary injury and improve cognitive performance following head trauma in two different preclinical models; (2) we will discover neuroprotective compounds that will be promising preclinical leads to treat TBI by a novel approach; and (3) we will generate tool compounds for identification of new pathways that regulate neuronal and oligodendrocyte survival and function, thereby leading to a better understanding of neurodegenerative processes.*

INNOVATION

All efforts over the past 25 years to develop a drug to treat TBI have failed. Although multiple contributing factors are involved, we believe numerous failed approaches resulted from insufficient "preclinical" rigor, which can be problematic in a complex, multi-faceted injury environment. For example, many studies have employed a single gender and TBI model and focused on a single pathological pathway. *Our approach is innovative because we will address these limitations by examining multipotential[5] compounds in both male and female mice using two injury models: blast and controlled cortical impact (CCI) injury.* Sig2R was first identified in 1990,[6] and it is implicated in cell proliferation, regulation of cytosolic calcium (Ca^{2+}) levels, and cellular processes related to CNS disorders.[7] We and others have discovered that Sig2R binding ligands are neuroprotective,[8,9] and we have shown that such compounds reduce levels of proinflammatory cytokines and regulate intracellular Ca^{2+} concentration,[8] as well as affect synaptic plasticity (unpublished). Although previous attempts by other groups to clone Sig2R failed,[10,11] we recently isolated, cloned, and identified it as transmembrane protein 97 (TMEM97) (see Aim 1, *Justification and Feasibility*).[12] The molecular function of TMEM97, whose sequence identity in rodents and humans is 81% (NCBI Blast), is not well understood, but it is believed to be involved in cholesterol trafficking and homeostasis.[13] In accord with what is known about Sig2R,[7a] TMEM97 is upregulated in cancer cells,[14] and it is expressed in various brain regions in rodents and humans, including the cortex, hippocampus, cerebellum, olfactory bulb, and the subventricular zone.[15] Little is known about the function of Sig2R/TMEM97, the term we will generally use herein, in the CNS, so *another innovative aspect of our approach is that we will use small molecules to modulate a biological target that has never been associated with TBI.* Our preliminary studies (see Aims 1–3, *Justification and Feasibility*) suggest this is a viable strategy because we have already shown that neuroprotective Sig2R/TMEM97 binding ligands improve performance in learning and memory tasks in two animal models of TBI. We recognize that other neuroprotective candidates have failed in the clinic[16] or are in early stages of development.[17–19] However, *our approach is unique because it comprises a number of pathological processes relevant to TBI, not just neuroprotection.* Examining all of the pathways Sig2R/TMEM97 mediates (*vide supra*) is beyond the scope of this proposal, but *we will test our novel hypothesis that Sig2R/TMEM97 ligands affect adult neurogenesis*, which has a significant impact on stabilizing the injury microenvironment in the cortex and hippocampus.[20] *A broader innovative feature of the proposed research is that validating Sig2R/TMEM97 as a new target for treating neurodegeneration and cognitive deficits sets the stage for applications to other neurological disorders and conditions.*

APPROACH

Overview: Modulating Sig2R/TMEM97 is a unique and promising new approach for treating patients after TBI. This novel hypothesis is based on our findings that strongly suggest that biological processes mediated by Sig2R/TMEM97 play key roles in neuron survival, as well as in behavior, learning, and memory after TBI. We have demonstrated that Sig2R/TMEM97 modulators can be neuroprotective and regulate intracellular Ca^{2+} concentration.[8] We have also shown that these agents reduce levels of proinflammatory cytokines and enhance cognitive performance in symptomatic, transgenic Thy-1 $hAPP^{Lond/Swe+}$ (Tg) mice, a standard animal model used to assess adverse effects of Aβ.[8] In unpublished work with Dan Johnston (The University of Texas), we have discovered that different Sig2R/TMEM97 modulators can have opposing effects on long-term depression (LTD) and potentiation (LTP) in neurons. These synaptic plasticity processes are critical to learning and memory.[21] Furthermore, in proof-of-principle studies relevant to TBI, we have shown that Sig2R/TMEM97 modulators significantly improve learning and memory in mice after two forms of TBI: blast-mediated and controlled cortical impact (CCI) injury. One of these compounds reduces both axonal degeneration and oligodendrocyte death after brain injury. *A significant strength of our approach is that we will use two TBI models that mimic both acute and chronic neurological deficits seen in patients,*[22] *so we will be able to examine temporspatial alterations in injury progression and recovery based on the specific characteristics of each model, and then apply overlapping functional assessments for comparisons.*

Our overall goal is to identify at least one selective and orally-bioavailable Sig2R/TMEM97 modulator that mitigates secondary nerve injury *and* enhances cognitive performance after brain injury for advancement into preclinical studies to treat TBI in humans. These objectives will be achieved in two phases by pursuing three Specific Aims. In Aim 1, we will design and synthesize new Sig2R/TMEM97 modulators having different scaffolds. We will then determine their receptor binding profiles, functional activities, and neuroprotective properties in order to identify neuroprotective compounds that have high-affinity (K_i ≤25 nM) for Sig2R/TMEM97 and high selectivity relative to Sig1R (≥25:1) and non-sigma CNS proteins (≥50:1). Standard assays to assess metabolic stability, permeability, efflux, brain and plasma protein binding, and CYP 450 inhibition will be performed for the more promising compounds. Preliminary pharmacokinetic (PK) profiles and absorption-distribution-metabolism-excretion (ADME) properties will be obtained for those compounds judged most likely to be orally-bioavailable based upon physicochemical properties. The structure-activity-relationship (SAR) data that emerges will be used in an iterative process that will deliver candidates for animal studies. We will identify at least three leads during Phase I and a total of six within the first year of the Phase II study. In Aim 2, we will evaluate our Sig2R/TMEM97 modulators in animal studies that will include: (1) screening the six most promising Sig2R/TMEM97 modulators in single dose studies using two brain injury models; (2) performing comprehensive PK analysis of the best candidate; and (3) characterizing the efficacy of this lead candidate in dose-response studies in the same two animal models of TBI to assess metrics of both cellular and functional outcomes. In Aim 3, we will perform quantitative autoradiography imaging studies using our lead Sig2R/TMEM97 modulator in order to characterize its binding to Sig2R/TMEM97 in brains harvested from uninjured and injured, as well as untreated and treated, mice from each of the injury models. This holistic approach is expected to lead to the identification of at least one drug-like, Sig2R/TMEM97 modulator that can be advanced as a potential therapeutic agent to treat TBI by a novel mechanism of action.

PHASE I – AIM 1. *Identify neuroprotective Sig2R/TMEM97 modulators with improved ADME/PK profiles.*

Introduction. We have shown in published and unpublished work that selective Sig2R/TMEM97 ligands are neuroprotective, mediate intracellular Ca^{2+} levels, reduce proinflammatory cytokines, modulate synaptic plasticity, and improve function in animal models of TBI. The *objective* of this aim is to prepare new selective Sig2R/TMEM97 ligands that are neuroprotective, cross the blood-brain barrier, and have good ADME/PK properties when administered orally. Our overall *approach* will involve: (1) using SAR data for current Sig2R/TMEM97 binders to design and synthesize new analogs predicted to have properties that align with CNS drugs; (2) determining receptor binding profiles; (3) determining functional activity; (4) assessing neuroprotective properties; (5) characterizing *in vitro* ADME profiles; and (6) performing preliminary PK analyses. *Notably, the overall process will be iterative with the developing SAR informing the design of new compounds.* We *expect* to identify novel Sig2R/TMEM97 modulators for animal studies in Aim 2 and target engagement studies in Aim 3. The *rationale* for these studies is that we will determine the pharmacophoric features required for potency, selectivity, and functional and neuroprotective activity, while also improving ADME/PK properties. The *positive impact* of these studies is that we will identify orally-bioavailable, neuroprotective compounds that can be used to validate a fundamentally new approach to treat patients with TBI.

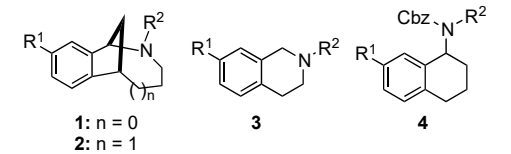


Fig. 1. Scaffolds of Sig2R/TMEM97 ligands.

Justification and Feasibility. We have prepared collections of substituted heterocycles[23,24] and submitted selected compounds to the NIH Psychoactive Drug Screening Program (PDSP) for screening against a panel of CNS targets. We discovered that some norbenzomorphans **1** and homonorbenzomorphans **2** (*all compounds are racemic unless otherwise indicated*) and their bicyclic analogs **3** and **4** (Fig. 1) exhibit high affinity and selectivity for Sig2R in radioligand binding assays.[25] Although the Sig1R was known to be involved in CNS pathophysiology, little was known about the role of Sig2R in CNS diseases and disorders.[26] We thus initiated a program to characterize the effects of modulating Sig2R on neurodegenerative conditions.

Sig2R had not been cloned when we started our work, so a key step in pursuing it as a therapeutic target was to clone it, and in collaboration with Andrew Kruse (Harvard University), we identified Sig2R as TMEM97.[12] Briefly, a high affinity, selective Sig2R binding ligand related to **4** (Fig. 1) was used to capture candidate proteins that were then sequenced by high-resolution mass spectrometry (HRMS). These were expressed in HEK293 cells, but only expression of TMEM97 resulted in increased binding of [^{3}H]-di-*o*-tolylguanidine [(^{3}H)DTG], which is highly selective for sigma receptors.[6] TMEM97 was then expressed in *Sf9* insect cells, which lack an endogenous TMEM97 gene and show no significant (^{3}H)DTG binding signal. Expression of TMEM97 resulted in the appearance of saturable (^{3}H)DTG binding with an affinity corresponding to literature values for its binding to Sig2R. Competition binding experiments with known Sig2R ligands show their binding affinities for TMEM97 are similar to reported values for Sig2R binding. Finally, a large reduction in (^{3}H)DTG binding was observed after siRNA knockdown of Tmem97 in PC-12 cells, a classic Sig2R expressing cell line*. Identifying Sig2R as TMEM97, which is referred to herein as Sig2R/TMEM97, represents a major breakthrough that opens the door to mechanistic and biochemical studies that were previously impossible.*

In order to assess whether compounds that bind to Sig2R/TMEM97 are neuroprotective, we collaborated with J. Pierce-Shimomura (The University of Texas; see biosketch and letter) using his novel *C. elegans* model of neurodegeneration. These worms carry a single copy of the human APP gene (SC_APP) and display age-dependent degeneration of cholinergic neurons.[8,27] A number of Sig2R ligands were identified that markedly decrease neurodegeneration. Gene knockout or knockdown studies are useful strategies to study mechanism, but at the time this work was done, we had not yet cloned Sig2R/TMEM97. However, it had been suggested that the Sig2R/TMEM97 receptor binding site was in a multi-protein complex containing the progesterone receptor membrane component 1 (PGRMC1),[10,28] a heme binding protein that is upregulated after brain injury[29] and is involved in cholesterol synthesis[30] and neuroprotection.[8,9] We therefore performed experiments in which expression of *vem-1*, the worm ortholog of PGRMC1, was knocked down by RNAi (Fig. 2). **SAS-0132** (Fig. 3) reduces neurodegeneration in transgenic SC_APP *C. elegans,* but because it provides no further neuroprotection in animals with reduced *vem-1* expression, neuroprotection arises from inhibiting a *vem-1* (*i.e.,* PGRMC1) pathway (Fig. 2). *These findings are the first to show that Sig2R/TMEM97 binding ligands have downstream effects on a PGRMC1-mediated pathway.* There is no direct ortholog of Sig2R/TMEM97 in *C. elegans,* but Y38H6C.16 has about 30% sequence similarity, and predicted secondary structures of Y38H6C.16 and Sig2R/TMEM97 are conserved. A D-residue in Y38H6C.16 corresponds to the essential D56 in Sig2R/TMEM97, and although no D-residue aligns directly with D29 in Sig2R/TMEM97, a D-residue is in the correct region. Hence, we believe Y38H6C.16 is a putative binding site for Sig2R/TMEM97 ligands in *C. elegans*, and this hypothesis is being examined in ancillary studies. *The absence of a direct ortholog of TMEM97 in C. elegans notwithstanding, we find this model is predictive of neuroprotection*.

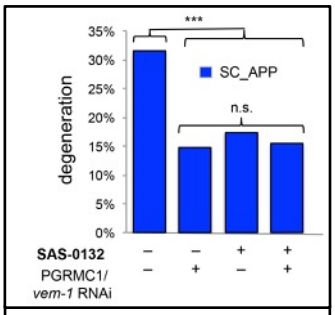


Fig. 2. Cholinergic neuron degeneration in SC_APP treated with *vem-1*-directed RNAi and/or **SAS-0132** shows that **SAS-0132** works by inhibiting *vem-1*-mediated pathway. ***p<0.001

Screening about 60 racemic analogs of **1**–**4** in receptor binding assays at the NIH PDSP led to the discovery of new potent and selective Sig2R/TMEM97 ligands (Fig. 3). All of these compounds satisfy the prevailing pharmacophoric model,[31] but comparing structures suggests there are two possible orientations for the (homo)norbenzomorphan ring in the binding site. Compounds **SAS-0132**, **DKR-1516**, and **6** are highly neuroprotective in transgenic *C. elegans*. Although we do not yet have a full understanding of the structural requirements for neuroprotective activity, *it is notable that all N-substituted piperazine analogs of **SAS-0132** tested thus far are neuroprotective.* Functional activity was assigned to some of these ligands using a protocol developed by Zeng,[32] wherein the cytotoxicity on the EMT-6 cancer cell line of a compound is compared with that of siramesine, a Sig2R/TMEM97 agonist. A ligand is considered to be an antagonist if it displays cytotoxicity <10% that of siramesine. However, because so little is known about the biological function of Sig2R/TMEM97, the terms "antagonist" and "agonist" should be used with caution. Accordingly, we will generally refer to these compounds as Sig2R/TMEM97 modulators. Recognizing this caveat, norbenzomorphans **SAS-0132, DKR-1516**, **6, 7**, and the homonorbenzomorphan **9** are **"**partial agonists", whereas **DKR-1051** is an "agonist". Other compounds in Fig. 3 have not yet been evaluated in this assay.

SAS-0132 and **DKR-1516** emerged as attractive candidates for further study. **SAS-0132** is ~9-fold selective for Sig2R/TMEM97 versus Sig1R, and it exhibits low off-target affinity (K_i >1 μM) for many CNS proteins, including most opioid, adrenergic, serotonergic, cannabinoid, nicotinic and muscarinic acetylcholine, and *N*-methyl D-aspartate receptors, as well as the neurotransmitter transporters DAT, NET, and SERT, and several ion channels (L-type Ca^{+2}, hERG, and Nav1.5). **DKR-1516** is more selective for Sig2R/TMEM97 relative to Sig1R and has lower off-target affinities for other CNS proteins than **SAS-0132.** Although the enantiomers of **SAS-0132** are not readily separable, we resolved **DKR-1516** and found that the 1*S*,5*R*-isomer binds with about 7-fold higher affinity for Sig2R/TMEM97 than the 1*R*,5*S*-isomer (2.7 nM vs 20 nM).

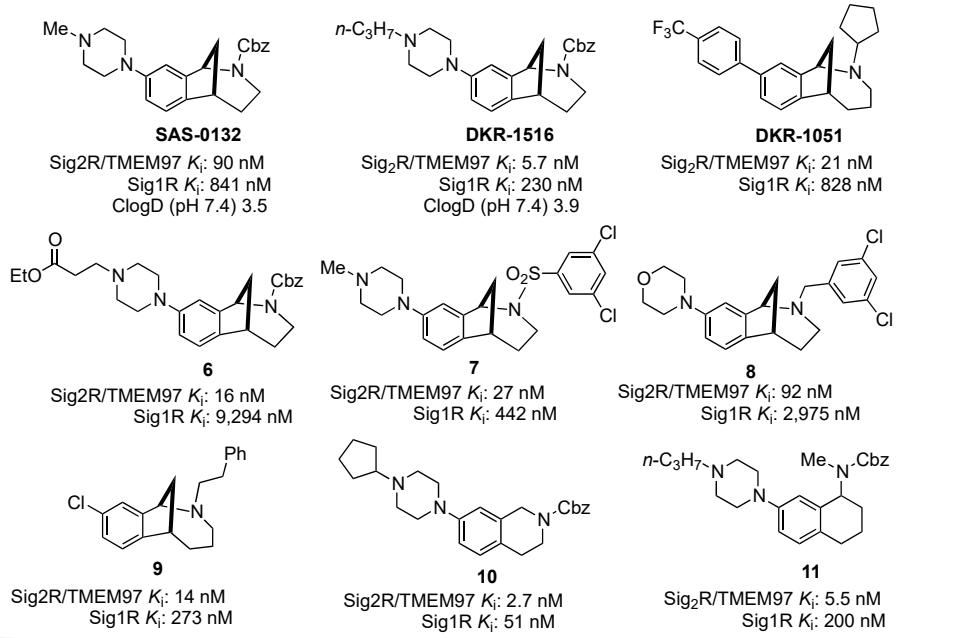


Fig. 3. Representative Sig2R/TMEM97 ligands and their Sig2R/TMEM97-Sig1R affinities.

Available PK data for **SAS-0132** and **DKR-1516** show that they achieve high plasma and brain concentrations after subcutaneous (SC) and intravenous (IV) dosing, but not after oral (PO) administration. In order to elucidate possible origin(s) of their low PO bioavailabilities, *in vitro* ADME studies of **SAS-0132** and **DKR-1516** were performed (Quintara Discovery). Although both compounds have desirable solubility properties, the short $t_{1/2}$ of **SAS-0132** and **DKR-1516** in mouse and human liver microsomes (~3 and 11 min, respectively) suggest that first pass metabolism is likely an issue. In a follow-up study with mouse liver microsomes, we found that **SAS-0132** and **DKR-1516** primarily undergo *N*-dealkylation and *p*-hydroxylation of the phenyl group of the Cbz moiety; *N*-oxidation and oxidation of aromatic ring of the norbenzomorphan ring may also occur, albeit to a lesser extent. **SAS-0132** and **DKR-1516** also have favorable *in vitro* permeability parameters (P_{app} >15x10^{-6} cm/s) and are not substrates for P-gp efflux (efflux ratios 0.9 and 0.6, respectively). **DKR-1516** achieves brain levels of ~40 nM and a favorable brain-to-plasma ratio (3.5:1) at 4 h post 3 mg/kg intraperitoneal (IP) administration. Free levels of **DKR-1516** in the brain (B_u) and plasma (P_u) are about 0.4% and 0.9% (B_u/P_u = 0.44), respectively. In a preliminary PK study (3 mg/kg, IP, four time points), **DKR-1516** exhibits a half-life ($t_{1/2}$) of 1.5 h, maximum plasma concentration (C_{max}) of 0.2 μM, and area under curve ($AUC_{0\text{-}inf}$) of 0.23 μM•h. **SAS-0132** and **DKR-1516** have not shown any toxic effects in animals. For example, no detrimental effects in blood chemistry biomarkers or histopathology of the brain and vital organs were observed after dosing animals for 60 days with **SAS-0132** (SC, 10 mg/kg/d). The maximum tolerated dose (MTD), commonly referred to as the "no adverse effect level" (NOAEL), of **SAS-0132** in mice is >100 mg/kg/d (SC) for 14 days. In efficacy studies (see Specific Aim 2, *Justification and Feasibility*), both **SAS-0132** and **DKR-1516** enhance functional performance in mice after blast injury following IP administration. Notably, **DKR-1516** also improves performance, reduces axon degeneration, and improves oligodendrocyte survival after CCI injury.

Research Design.

(1) Design and synthesize novel Sig2R/TMEM97 modulators. The overall goal of this aim is to identify potent and selective Sig2R/TMEM97 modulators for use in the proposed animal (Aim 2) and target engagement (Aim 3) studies. We will use existing and emerging SAR data to design and prepare new Sig2R/TMEM97 binding ligands with particular *focus on improving selectivity relative to other CNS proteins and molecular properties associated with favorable ADME and PK attributes for CNS drugs.*[33–36] Namely, we will focus on analogs of **DKR-1516** that have comparable aqueous solubility, ClogD (pH 7.4) values of ~2 to 3.5, and total polar surface area (TPSA) <70 $Å^2$ in order to favor brain penetration, increase free levels in the brain (*i.e.,* non-specific protein binding), and reduce the likelihood of significant hERG channel affinity. Because there are no significant differences in efficacy in animal studies for the enantiomers of **DKR-1516** (Aim 2, *Justification and Feasibility*), we will use racemic compounds. *This will be an iterative process in which compounds must meet the sequential criteria set forth in parts 2–6 to be advanced.*

As a guiding strategy toward enhancing oral bioavailability, we will first identify analogs that meet the criteria of parts 2–4 of this aim and are expected to have improved liver microsomal stability, while maintaining other favorable physicochemical properties for CNS drugs. *N*-Dealkylation and *N*-oxidation of cyclic tertiary

amines by cytochrome P450 (CYP 450) enzymes can be suppressed by increasing steric bulk and placing electronegative atoms proximal to the nitrogen atom, whereas oxidation of aromatic rings can be decreased by making the ring less electron rich and/or blocking the site of oxidation.[36] Secondary amines are generally more metabolically stable than tertiary amines. Indeed, **12** (Fig. 4, R^1=R^2=H_2, R^3=Cbz), which has a $t_{1/2}$ in mouse liver microsomes of ~40 min, is neuroprotective and has high affinity (K_i= 25 nM) and selectivity (65:1) for Sig2R/TMEM97 versus Sig1R. Accordingly, we will first prepare about 50 exemplary compounds of the general form **12** and **13** (Fig. 4). Analogs of **12** (R^1=R^2=H_2, Me_2) will elucidate the SAR associated with the *gem*-dimethyl group and the R^3 substituent. Compounds **12** (R^3=*p*-F-Cbz, SO_2Ar, COAr, *etc.*) will not undergo oxidation of the phenyl ring of the *N*-Cbz group. We have limited SAR for varying R^3, but the activity of **7** (Fig. 4) shows that this is a valid approach. The set of compounds **13** with a metabolically stable R^3 group are designed to assess the effects of the *gem*-dimethyl moiety and of electron withdrawing groups on the *N*-alkyl substituent. We will then use the SAR from these experiments and focus on the more promising substitutions (*i.e.,* R^1, R^2, and R^3) to design a set of about 25 exemplary compounds **14**–**16**, which will be more resistant toward oxidation of the norbenzomorphan ring.

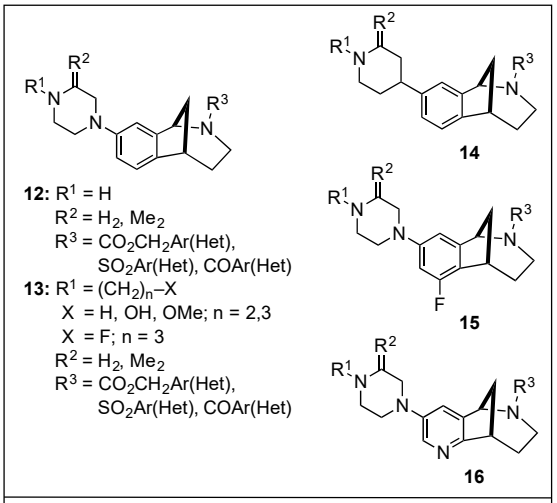


Fig. 4. Exemplary analogs of **DKR-1516** likely to exhibit improved metabolic stability.

(2) Determine receptor binding profiles of new compounds. New compounds will be submitted to the PDSP (see supporting letter from Professor B. Roth) for screening at Sig2R/TMEM97 and Sig1R. In order to identify those ligands with low binding to other CNS proteins, those Sig2R/TMEM97 binding ligands with K_i ≤25 nM and selectivity of ≥25:1 over Sig1R will be advanced for profiling against a panel of about 45 CNS proteins (*e.g.,* ORs, DRs, 5-HTRs, CBs, nAChR, mAChR, ARs, NMDAR), neurotransmitter transporters (*e.g.,* DAT, NET, SERT), and ion channels (*e.g.,* L-type Ca^{2+}, hERG, Nav1.5).[37] Toward maximizing the therapeutic index of Sig2R/TMEM97 modulators, preferred compounds will have a 50- to 100-fold preference for Sig2R/TMEM97 relative to other targets and >500-fold selectivity for targets known to be associated with adverse side effects and drug reactions.[38] For example, hERG, $5HT_{2B}$ (agonism), and Nav1.5 have been implicated in cardiac abnormalities, so only those modulators showing weak affinity for hERG (K_i >10 μM), $5HT_{2B}$ (K_i >1 μM), and Nav1.5 (>10 μM) will be eligible for advancement.

(3) Determine functional activity of most potent and selective Sig2R/TMEM97 modulators. We will continue to use the cell-based assay reported by Zeng[32] to determine functional activities of Sig2R/TMEM97 binding ligands meeting the criteria of part 2. We have found that compounds having a relative cytotoxicity ranging from <10 to ~50% of siramesine (*i.e.,* "antagonists" and "partial agonists") in this assay are neuroprotective, so only those modulators fitting this criterion will be advanced.

(4) Determine neuroprotective activity of more promising "antagonists" and "partial agonists". We will continue to collaborate with Pierce-Shimomura using his *C. elegans* model of hAPP-related neurodegeneration to screen Sig2R/TMEM97 modulators for neuroprotective activity *in vivo.*[27] Only compounds that exhibit neuroprotective activity comparable to **DKR-1516** will be advanced.

(5) In vitro ADME studies. Compounds meeting the criteria of parts 2–4 will be sent to Quintara Discovery for *in vitro* assessments. Liver (mouse, rat, human) microsomal stability assays will be performed, and candidates having half-lives ≥60 min in at least two liver microsomal preparations will be preferred. Cell permeability will be evaluated using Madin-Darby canine kidney (MDCK) cells transfected with multi-drug resistant gene 1 (MDR1)-MDR1 cells to assess *in vitro* absorption properties and the potential role of P-glycoprotein (P-gp) transport. Because MDCK-MDR1 permeability correlates well with brain/plasma exposure and area under the curve (AUC), it is a better predictor of brain penetration than other assays.[39] Compounds having P_{app} values of >$10x10^{-6}$ cm/s and efflux ratios <2 will be advanced. We will then determine plasma protein and brain tissue binding (mouse), and those compounds having unbound levels of >1% in the brain will be preferred. However, **DKR-1516**, which is efficacious at doses ranging from 0.3–3.0 mg/kg, is 0.4% free in the brain, so free levels can be lower than 1%. Nevertheless, more polar analogs are expected to have improved unbound levels.

(6) Preliminary PK analysis. The preliminary PK analysis of more promising Sig2R/TMEM97 modulators will be performed by Quintara Discovery following the same approach used for **DKR-1516**. Briefly, mice will be dosed (PO) with approximately 3–10 mg/kg of modulator, and blood will be collected at four time points (5, 15, 60, and 240 min) with brain tissue being collected after terminal blood collection. Concentrations in plasma and brain tissues will be determined to give values for $t_{1/2}$, C_{max}, AUC_{0-inf}. Based upon data for **DKR-1516** (brain levels 4 h post 3 mg/kg IP dose ~40 nM; $t_{1/2}$ = 1.5 h, C_{max} = 0.2 μM, and AUC_{IP} = 0.23 μM•h), preferred compounds will achieve brain levels >100 nM and values for $t_{1/2}$ of >3 h, C_{max} of >1 μM, and AUC_{0-inf} of 1–2 μM•h.

We will perform CYP inhibition studies against the key CYP isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 for those Sig2R/TMEM97 modulators selected for efficacy studies, so we can focus on candidates having IC_{50}s >10 μM for all isoforms.

Pitfalls and Alternatives. We do not anticipate significant problems, but if planned synthetic routes to specific compounds prove troublesome, we will design new ones. We may need to prepare more than the projected ~75 compounds in order to achieve our Phase I goal of identifying at least three new Sig2R/TMEM97 modulators meeting the criteria in parts 2–6; we have the personnel, resources, and experience to do so.

Criteria for Phase II advancement and Fast-track justification.

We know IP administration of **DKR-1516** to mice after blast and CCI injury increases oligodendrocyte survival, reduces axon degeneration, and improves function. It is thus an excellent starting point to improve ADME/PK properties. *As the **Milestone** for Phase I studies, we will synthesize at least 75 new norbenzomorphan analogs of **DKR-1516** and identify at least three novel candidates related to **12–16** that meet Phase I criteria and can be advanced to animal studies in Phase II.* Our extensive experience coupled with our highly promising preliminary results strongly suggest that we will achieve the Phase I goals in one year, thus justifying our Fast-track application to develop a different approach to treat TBI in humans. A simplified overview of the project is given in Fig. 5.

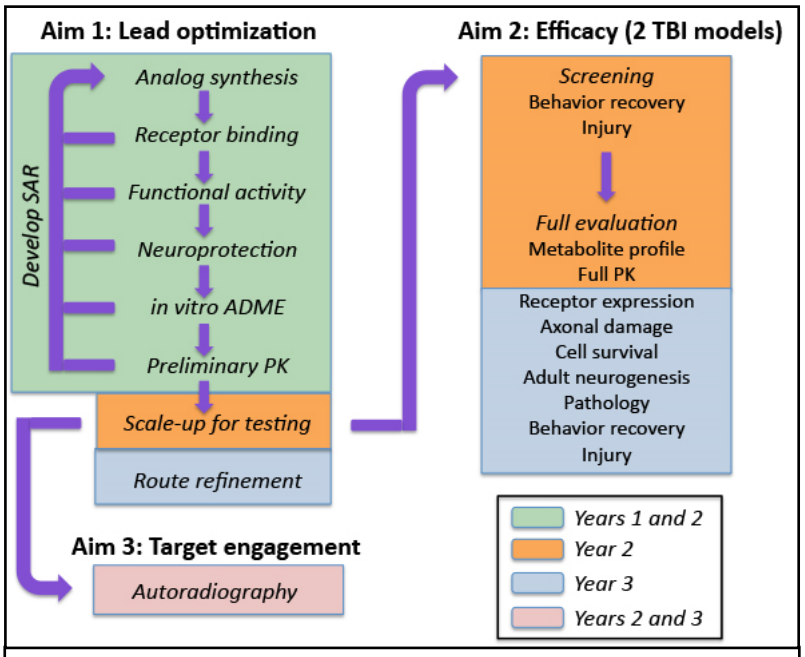
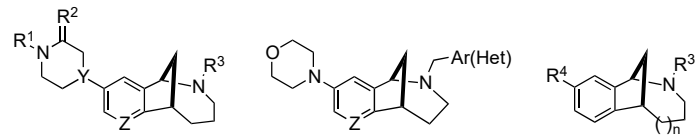


Fig. 5. Overview and approximate timeline for the entire project. Phase I will be completed in one year, and a set of orally bioavailable analogs of norbenzomorphan **DKR-1516** will be ready for advancement to Phase II (Aims 2,3). Phase II will require two years during which time modulators having significantly different structures than **DKR-1516** will be prepared, and animal efficacy and imaging studies will be completed, thereby setting the stage for toxicity studies and a pre-IND meeting with the FDA.

PHASE II – AIM 1 *continued*.

(7) *Characterize profiles* of *Sig2R/TMEM97 modulators with alternate scaffolds.* It is critical to identify candidates having structural features different from the initial leads, which are largely piperazino norbenzomorphans, because such compounds are likely to have different off-target profiles and ADME/PK properties. New compounds will be evaluated on the basis of the criteria outlined in parts 2–6 of Aim 1 in Phase I studies. We will thus use the emerging SAR data for **12**–**16** and focus on the more promising substituents R^1–R^3 to prepare a collection of approximately 75 homonorbenzomorphans **17** (Fig. 6). We will also synthesize about 15 compounds of the general structure **18** (Fig. 6) in order to examine analogs of the highly selective Sig2R/TMEM97 binding ligand **8** (Fig. 4). Because compounds lacking a basic amino substituent on the aromatic ring of the homonorbenzomorphan (*e.g.,* **DKR-1051** and **9** (Fig. 4) are attractive Sig2R/TMEM97 modulators, we will make a series of about 20 compounds represented by **19** (Fig. 6).



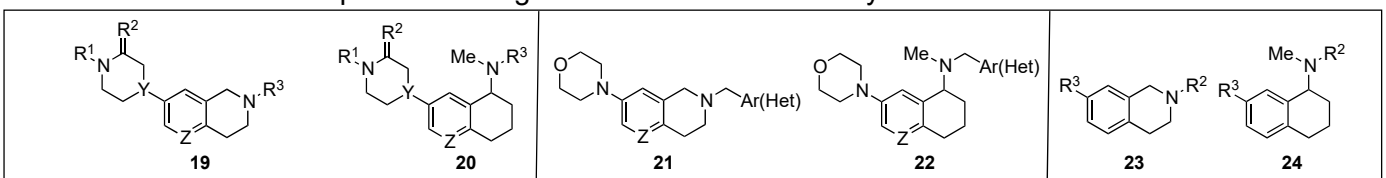
17: R^1 = H, Me, or $(CH_2)_n$–X
X = H, OH, OMe, F; n = 2,3;
Y = CH or N; Z = CH, CF or N
R^2 = H_2, Me_2
R^3 = $CO_2CH_2Ar(Het)$, $SO_2Ar(Het)$, COAr(Het), etc

18: Z = CH, CF or N

19: n = 0, 1
R^3 = alkyl, cycloalkyl, functionalized alkyl, $(CH_2)_nAr(Het)$
R^4 = Hal, Ar, Het

Fig. 6. Other exemplary (homo)norbenzomorphan analogs of **DKR-1516.**

Preliminary binding data for tetrahydroisoquinolines (*e.g.,* **10**, Fig. 3) and aminotetralins (*e.g.,* **11**, Fig. 3) show that derivatives of compounds having these scaffolds are worthy of further examination. Because the lim-



19 **20**

R^1 = H, Me, or $(CH_2)_n$–X; X = H, OH, OMe, F; n = 2,3;
Y = CH or N; Z = CH, CF or N
R^2 = H_2, Me_2
R^3 = $CO_2CH_2Ar(Het)$, $SO_2Ar(Het)$, COAr(Het), etc

21 **22**

Z = CH, CF or N

23 **24**

R^2 = alkyl(functionalized), cycloalkyl, $(CH_2)_nAr(Het)$
R^3 = Hal, Ar, Het

Fig. 7. Exemplary tetrahydroisoquinoline and aminotetralin analogs.

ited SAR data that is available show parallels in the binding profiles of these compounds with those of their norbenzomorphan analogs, we will rely on the SAR obtained for **12**–**19** to establish priorities for preparing about 50 tetrahydroisoquinolines and aminotetralins of the general form **19**–**24** (Fig. 7).

(8) Scale-up synthesis of Sig2R/TMEM97 modulators. The six most promising candidates emerging from parts 1–7 will be prepared in sufficient quantities for screening for injury and behavior recovery in blast and CCI injury models (Aim 2, *Research Design*, part 1). The selected lead compound will then be synthesized in quantities sufficient to complete full PK and efficacy evaluation (Aim 2, *Research Design*, parts 2,3). We will synthesize an analog of this lead that has either a C–C double bond or two bromine atoms on an aromatic ring so it can be isotopically labeled by catalytic tritiation and used in the autoradiography studies in Aim 3.

(9) Modify synthesis of lead Sig2R/TMEM97 modulator for translational studies. The current synthetic strategy for preparing new Sig2R/TMEM97 modulators for screening is specifically designed to facilitate the synthesis of small quantities of analogs from advanced intermediates. It is not the most efficient route to a single compound. There are also late stage transition metal-catalyzed reactions, which are undesirable for preparing active pharmaceutical ingredients (API) according to good manufacturing practice (GMP). Accordingly, we will develop an alternate synthetic approach that can be scaled up and performed in a GMP facility to prepare the quantities needed for toxicology and other preclinical studies.

Expected Outcomes. We expect to identify and synthesize quantities for testing of at least six new Sig2R/TMEM97 modulators that meet the criteria set forth in parts 2–6 that can be advanced to the animal studies in Aim 2. We will synthesize quantities of modulators for efficacy and autoradiography studies and develop an alternate synthetic route to our lead that is suitable for scale-up to prepare API in accord with GMP.

Pitfalls and Alternatives. If six new compounds for efficacy testing in animal studies do not initially emerge from Aim 1, we will synthesize additional candidates related to **12**–**24** using available SAR data.

AIM 2. ***Evaluate effects of Sig2R/TMEM97 modulators in two animal models of TBI.***

Introduction. Our critical proof-of-principle experiments show that treating mice with Sig2R/TMEM97 modulators after blast and CCI brain injury reduces axonal degeneration and oligodendrocyte death and enhances performance in learning and memory tasks to levels seen in sham-injury control animals. Because the sequence identity of Sig2R/TMEM97 in mice and humans is 81% (NIH Blast), we believe efficacy studies in mice provide a valid model for the human condition and that Sig2R/TMEM97 is a fundamentally new and promising biological target for treatment of TBI in humans. The *objective* of this Aim is to identify a promising, orally-bioavailable Sig2R/TMEM97 modulator that mitigates secondary nerve damage, improves motor function and cognitive performance of mice after brain injury, and can be advanced to preclinical toxicity studies. Our *approach* will involve: (1) screening the six most promising Sig2R/TMEM97 modulators identified in Aim 1 in single-dose (PO) studies to assess cell survival and behavioral outcomes in male and female mice using blast and CCI injury models; (2) determining the PK parameters (PO) of the best candidate; (3) performing dose-response (PO) studies of the best Sig2R/TMEM97 modulator in blast and CCI injury models to assess neurofunctional outcomes; (4) performing *ex vivo* studies to evaluate Sig2R/TMEM97 expression levels, tissue sparing, neuron/oligodendrocyte survival, axonal damage, gliosis, and adult neurogenesis. We will be able to examine temporospatial alterations in injury progression and recovery based on the specific characteristics of each of the two models and thus enable us to apply overlapping functional assessments for comparison. The *rationale* for these studies is that we will fully characterize the neurofunctional and pathophysiological effects of treating mice with a Sig2R/TMEM97 modulator after two different types of brain injury. The *positive impact* of these studies is that they will deliver an efficacious Sig2R/TMEM97 modulator for preclinical toxicology studies and enable a pre-IND meeting with the FDA for approval to further develop a drug to treat TBI in humans by a novel mechanism of action.

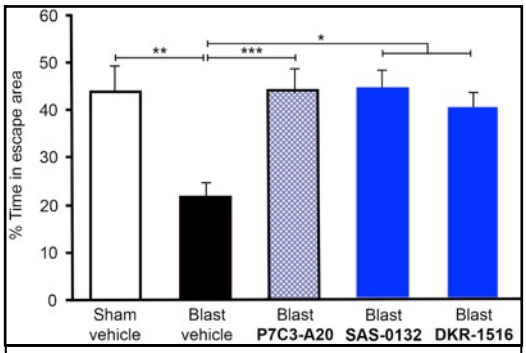


Fig. 8. Performance in Barnes maze test after blast injury. Animals treated with **DKR-1516** (10 mg/kg) and **SAS-0132** (10 mg/kg) performed comparable to control (Sham) and potent positive control **P7C3-A20** (10 mg/kg). *p<0.05, ** p<0.01, ***p<0.001.

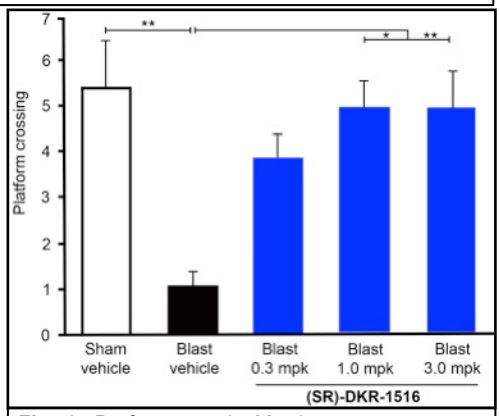


Fig. 9. Performance in Morris water maze test after blast injury. Blast animals dosed with 0.3, 1.0, and 3.0 mg/kg of **(1*S*,5*R*)-DKR-1516.**

Justification and Feasibility. The search for new drugs having novel mechanisms of action to treat TBI by intervening in the numerous biological pathways that ensue following initial injury has been largely unsuccessful, so it is critical to evaluate all promising possibilities. It is thus significant that we recently identified Sig2R/TMEM97 as a promis-

ing target for developing therapeutic agents to treat TBI by a mechanism of action fundamentally different from all previous methods. A key element of our approach is that we will use animal models of TBI that mimic both mild and moderate neurologic deficits that are seen in human patients.[22]

We collaborated with Andrew Pieper at the University of Iowa (see biosketch and supporting letter) to assess the effects of **SAS-0132** and **DKR-1516** in a mouse model of blast-induced TBI. Several reports demonstrate that the key consequences of human TBI are faithfully recapitulated in this model.[40] For example, Pieper and others have shown that blast-mediated TBI in mice causes visual, cognitive and motor dysfunction phenotypes that mirror blast-TBI damage in patients.[17a,40b] In key proof-of-principle studies, we discovered that animals treated with **SAS-0132** (10 mg/kg, IP) and **DKR-1516** (10 mg/kg, IP) after blast injury performed much better in the Barnes maze test of hippocampal-dependent spatial learning and memory than did vehicle-treated, injured animals (Fig. 8). Indeed, treated animals functioned on par with both the sham blast group (vehicle) and the injured group treated with the highly potent positive control **P7C3-A20**, a well-established, neuroprotective aminopropyl carbazole.[17] In a followup dose-response study with **(1*S*,5*R*)-DKR-1615**, and **(1*R*,5*S*)-DKR-1516**, treatment with as little as 0.3 mg/kg of **(1*S*,5*R*)-DKR-1615** significantly enhances performance of blast-injured mice in the Morris water maze test for hippocampal-dependent spatial learning and long-term memory (Fig. 9); **(1*R*,5*S*)-DKR-1516** was similarly effective (data not shown). Silver staining of cortical tissue shows **DKR-1516** markedly reduces axon degeneration after TBI (Fig. 10).

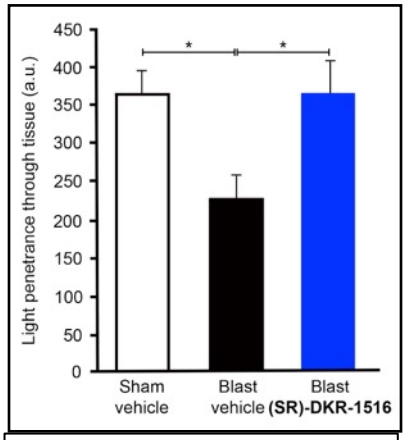


Fig. 10. Optical densitometry of visible light transmitted through silver-stained cortical brain tissue (n=5/group) used to quantify extent of axonal degeneration. The specific tissue area was manually delineated, and signal was quantified for 18 sections spaced 480 μM apart for each animal. A greater value correlates to protection and decreased axonal degeneration because increased light penetrance through the section results from less silver staining. Data are represented as mean ± SEM. *p < 0.05 compared to blast-injured animals treated with vehicle.

A second set of studies with Daniel Liebl at the University of Miami (see biosketch and supporting letter) was performed to assess the effects of **DKR-1516** after CCI injury. Over the past 10–15 years, the Liebl lab has developed and refined a moderate CCI injury model in mice in which temporospatial analysis of neuron and oligodendrocyte survival can be compared to anatomical and behavior outcome measures. Figure 11 shows CCI injury-induced loss of oligodendrocytes residing in the corpus callosum/external capsule (Fig. 11c) and the cortical penumbra (Fig. 11d). These temporospatial profiles are important for evaluating the timing of compound administration, and suggest that delivery prior to six hours post-injury (hpi) is important to maximize the protective effects in oligodendrocytes. Neuronal counts are pending. CCI injury also leads to behavioral deficits, in which pronounced losses in motor function (using Rotarod assessment) can be reversed following drug administration.[41] To examine whether the compound **DKR-1516** was effective in preserving cognitive/learning behavior after CCI-injury, we evaluated the efficacy of 3 mg/kg/day IP administration for six days post injury (dpi) and observed significant improvements in learning behavior using a novel object recognition (NOR) test. CCI injury results in reduced recognition of a novel object as compared with the familiar object (*i.e.,* Recognition index) at 7 dpi, which is reversed following **DKR-1516** administration (Fig. 12a). We also show that these improvements in learning and memory are associated with improved oligodendrocyte survival in the cortical white matter tracts that reside directly

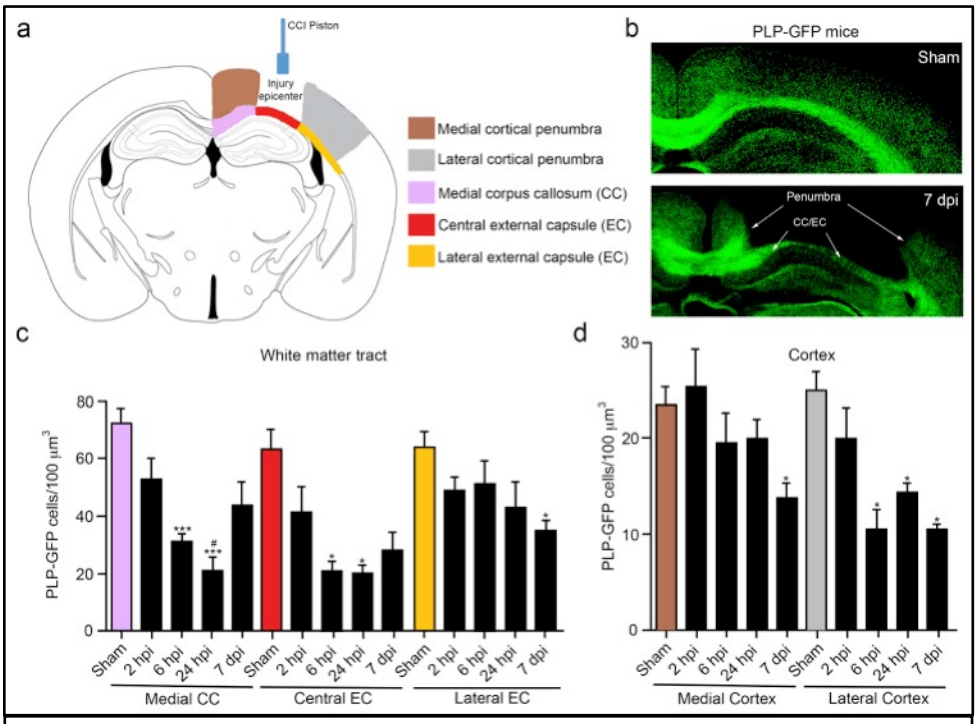


Fig. 11. CCI injury leads to temporospatial differences in OL cell loss. (a) Schematic of brain regions used for stereology. (b) PLP-GFP cortex, corpus callosum and external capsule in sham and CCI injured mice at 7 dpi. Stereological OL (c) and neuron (d) counts at 2, 6, 24 hpi and 7 dpi. n=8-14 per group. * compared to sham, # compared to 2 hpi. *,# p<0.05, ** p<0.01, *** p<0.001.

below the injury epicenter, where significant reductions in oligodendroctyes are observed following vehicle-treated CCI injury, *but not **DKR-1516**-treated CCI injured mice at 7 dpi (Fig. 12b).* Similar findings are observed in the corpus callosum (not shown), although greater numbers of mice are needed for all groups.

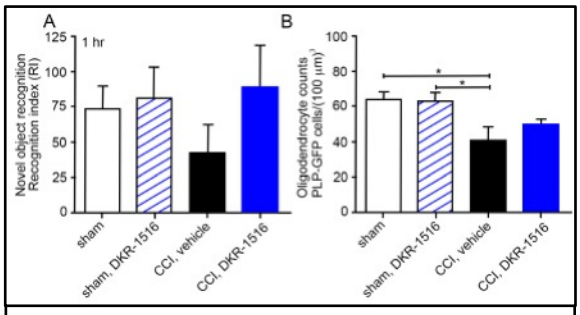


Fig. 12. DKR-1516 administration reverses CCI injury-induced deficits in NOR (a) and oligodendrocyte cell death (b). n=4-5/group; * p<0.05.

Collectively, previous findings in our lab coupled with these critical proof-of-principle studies show that Sig2R/TMEM97 modulators have excellent bioavailability and brain exposure, are non-toxic over extended periods of daily exposure, regulate intracellular Ca^{2+} levels, affect synaptic plasticity, reduce proinflammatory cytokines, reduce axon degeneration, improve oligodendrocyte survival, and enhance cognitive performance after brain injury in two different injury models. These results provide compelling support for the exciting potential of Sig2R/TMEM97 modulators as potential drugs to treat TBI by a new mechanistic pathway.

Research Design

*(1) Screen effects of six Sig2R/TMEM97 modulators in **blast** and **CCI injury** models.* The blast-injury model of TBI will be conducted in the Pieper laboratory, whereas the CCI model will be done in the Liebl laboratory. In order to identify a candidate for full evaluation, we will first screen the six most promising compounds emerging from Aim 1 in single-dose/day experiments. These will focus upon assessing the ability of each Sig2R/TMEM97 modulator to enhance behavioral recovery after injury in both the blast and CCI injury models, as well as axon integrity in the blast model and cell survival in the CCI injury model. During these experiments, we will monitor mice for any adverse neurological or health effects through standard observational procedures for preclinical studies in the Pieper and Liebl labs. The PK studies in Aim 1 will enable us to determine the dose (PO), and based upon our previous work we anticipate doses will be between 1–10 mg/kg/day.

*a. Assess the effects of six Sig2R/TMEM97 modulators on recovery in **blast injury** model.* We will screen the six most promising Sig2R/TMEM97 modulators by assessing behavioral outcomes (n=15/group, for each sex). Briefly, we will divide male and female C57Bl/6j mice into four groups (sham/vehicle, blast/vehicle, sham/compound, blast/compound). Blast injury will occur on day-0, and daily PO administration of test compounds and vehicle will begin one hour after injury and continue once daily for six consecutive days. On days 4 and 5 we will evaluate motor behavior using an open field test, which is part of the NOR training period. NOR will be tested on day-6 (1 h following training) and day-7 (24 h following training) to evaluate both short- and long-term recognition memory. NOR is a particularly attractive screening test since it does not require external motivation, reward or punishment, and it is thought to closely reflect parameters for the recognition memory test in human TBI patients.[42]

*b. Assess the effects of six Sig2R/TMEM97 modulators on injury and recovery in **CCI injury** model.* Using a CCI injury model, we will screen the same six Sig2R/TMEM97 modulators, as described for blast injury, and assess cell survival and behavior outcomes (n=15/group). Briefly, we will divide male and female C57Bl/6j mice into four groups (sham/vehicle, CCI/vehicle, sham/compound, CCI/compound). CCI injury (4 m/s, 0.5 mm depth) will occur on day-0, and daily PO administration of test compounds and vehicle will begin one hour after injury and continue once daily for six consecutive days. On days 4 and 5 we will evaluate motor behavior using an open field test, which is part of the NOR training period. As with the blast-model, NOR will be tested on day-6 (1 h following training) and day-7 (24 h following training) to evaluate both short- and long-term recognition memory. On day-7 we will harvest brain tissues in behaviorally tested animals for stereological counts of neurons and oligodendrocytes (see section 3d below).

(2) Determine metabolite and full PK profile of lead Sig2R/TMEM97 modulator. The metabolite profile of the lead will be characterized in rat, dog, monkey, and human to identify any differences in profiles in humans and in animals that will be used in toxicology studies. Detailed PK studies of the Sig2R/TMEM97 modulator having the best oral ADME/PK parameters and the best results in the single-dose animal injury studies (see part 1 above) will be performed by Quintara Discovery. Briefly, three escalating doses, which will be guided by the preliminary PK studies (see Aim 1, *Research Design*, part 6) of compound will be administered (PO) to C57Bl/6j mice (n=3/group). A 7-point PK curve will be generated by euthanizing mice at 5, 15, 30, 60, 120, 240 min, and 24 h after administration of the Sig2R/TMEM97 modulator at which times blood and brain tissue will be collected. Concentrations will be determined to give $t_{1/2}$, C_{max}, and AUC in both blood and brain tissue at different time points. The results of these studies will enable us to identify the range of daily oral doses for the dose-response studies in the two injury models, which will be conducted in parallel.

*(3) Fully characterize the effects of Sig2R/TMEM97 modulators on **blast** and **CCI** injury and recovery*. Having identified the optimal Sig2R/TMEM97 modulator, we will examine receptor expression, tissue sparing,

axonal damage, neuronal and oligodendrocyte survival, neurogenesis, and behavioral recovery between groups. If sex differences are observed between test compounds, we will continue to evaluate both male and female mice. If no differences are observed, we will limit our dose-response analysis to male mice. Indeed, a compound that shows efficacy in both sexes is optimal. With the exception of the expression studies, we will then examine all tissue and behavioral parameters using three doses based upon PK studies and varying overall by a factor of 10.

*a. Examine Sig2R/TMEM97 expression in **blast** and **CCI** injured tissues.* We will examine Sig2R/TMEM97 expression in sham, blast-injured, and CCI-injured brains at 1, 3 and 7 days post-injury (dpi) using Western blot analysis and immunohistochemistry (n=6/group). These time points were chosen based on the acute changes in cell survival, gliosis, tissue loss and neurogenesis described below. Western blot studies will be performed on cortical and hippocampal lysates using anti-Sig2R/TMEM97 antibodies, while immunohistochemistry studies will require serial cryostat sectioning throughout the region of injury. With CCI, this will be the region surrounding the site of impact. With blast-injury, this will be bilateral examination of serial sections in the anterior-posterior axis, as the blast wave affects all regions of the brain.[17a] Co-staining studies will employ neuronal (anti-NeuN) and OL (anti-CC1) antibodies to identify cellular localization. Based upon the Allen Brain Atlas, we anticipate finding Sig2R/TMEM97 mRNA in several regions of the naïve adult brain, including the cortex, hippocampus, and subventricular zone (SVZ).[15a]

*b. Evaluate effects of Sig2R/TMEM97 modulators on tissue pathology following **CCI** injury*. An overall assessment of cortical tissue sparing and gliosis will be performed with three drug doses (n=6/group) using protocols we previously developed.[20] Metamorph software (Molecular Devices) will be used to quantify the volume of remaining haemotoxylin and eosin (H&E) stained cortical tissue volume at 7 dpi as well as a more chronic recovery period of 28 dpi to determine whether acute affects are maintained over chronic periods. In these same animals, the volumetric area of astrocyte reactivity (*i.e.,* gliosis) in the cortex will also be measured using anti-GFAP antibodies. Brains will be fixed, serially sectioned at 50 μm, stained, and examined in a non-biased blind manner. Only the CCI injury model will be assessed in this capacity, since it contains defined injury borders as compared to the diffuse nature of the blast model.

*c. Evaluate effects of Sig2R/TMEM97 modulators on axonal damage following **blast** and **CCI** injury*. An overall assessment of axon degeneration will be performed at three compound doses (n=6/group) using protocols we previously developed.[17a] For blast injury, we will perform silver staining of serial sections in the anterior-posterior axis after behavioral testing is complete. Histologic examination with silver staining of brain tissue at this stage after TBI reveals prominent axonal degeneration in the absence of cell death or acute inflammation.[17a] Automated optical densitometry of silver-stained tissue is routinely used to quantify the magnitude of staining, such that greater impedance of light through the section reflects greater axonal degeneration.[17a] This method correlates with a previously established method of quantifying silver staining through manually delineating the area of interest and determining optical density of silver staining on the same section through NIH Image J software.[43] In the CCI injury model, serial sections of callosum/external capsule regions in and around the injury epicenter at 7 and 28 dpi will be sent to the Pieper Lab for analysis using a similar approach as described for the blast injured tissues.

*d. Evaluate effects of Sig2R/TMEM97 modulators on cell survival of neurons and oligodendrocytes following **CCI** injury.* We will quantify cell numbers using non-biased stereology in the cortical penumbra, corpus callosum/external capsule and hippocampus (DGC, CA1, and CA3 pyramidal cells) in sham and CCI injured mice at three drug doses to compare vehicle and drug treatment (n=8/group) as previously published.[41,44] Stereoinvestigator software (MBF Bioscience) will be used to quantify the numbers of neurons and oligodendrocytes at 7 and 28 dpi using fluorescent reporter mice [*i.e.,* Thy-1-YFP (neurons) and PLP-GFP (oligodendrocytes)] and/or immunohistochemistry. We will also quantify the number of TUNEL and/or activated caspase-3 positive cells at 1 and 3 dpi in neurons and oligodendrocytes using co-immunohistochemistry. We will only assess cell survival in the CCI injury model because temporospatial losses in cell numbers are well-defined (see Fig. 11), whereas they are not in the diffuse blast injury model.

*e. Examine the effects of Sig2R/TMEM97 modulators on adult neurogenesis following **blast** and **CCI** injury.* Because adult neurogenesis has a significant impact on stabilizing the injury microenvironment in both the cortex and hippocampus, we will examine the adult neurogenic response to CCI injury in the SVZ and hippocampus as previously published.[20,44a,45] We will evaluate neural stem/progenitor cell proliferation and survival to compare vehicle- and drug-treated sham with blast-injured and CCI-injured mice at three drug doses. Mice will be pulsed with 50 mg/kg of EdU on 1, 3, 5 dpi and sacrificed at 7 and 28 dpi for tissue analysis (n=8/group). For proliferation, serial sectioned brains will be stained for EdU and co-labeled with the neuroblast marker anti-double cortin (DCX) to quantify the number of proliferating neuroblasts at 7 dpi in SVZ tissues and the dentate gyrus of the hippocampus. For neuronal differentiation, we will examine the number of EdU positive cells that differentiate into NeuN positive neurons in the olfactory bulb and dentate gyrus at 28 dpi. For survival, we will examine the number of TUNEL positive-EdU labeled neuroblasts (DCX positive) and neurons (NeuN positive)

in the SVZ and dentate gyrus at 3 and 7 dpi. Non-biased StereoInvestigator analysis will be used for all quantitative measurements.

*f. Determine whether Sig2R/TMEM97 modulators enhance behavioral recovery after **blast** injury.* We will determine whether Sig2R/TMEM97 modulators improve functional recovery in sham and blast-injured mice using four behavioral assessments of sensorimotor function and learning behavior at three compound doses at 28 dpi. We will test two measures of sensorimotor function (*i.e.,* open field and Rotarod assessment) and two measures of cognitive/learning behavior (NOR and Morris water maze) to evaluate the effects of drug treatment (n=25/group) as previously published.[17a] Open field and NOR will be measured in the first week (as described above) together with Morris water maze at 13 dpi. Rotarod assessment will be performed on separate groups of animals (n=25/group) at 3, 7, 14 and 28 dpi since pre-injury training is required.

*g. Determine whether Sig2R/TMEM97 modulators enhance behavioral recovery after **CCI** injury.* We will determine whether Sig2R/TMEM97 modulators can improve functional recovery in CCI-injured mice using the same four behavioral assessments (n=15/group) as described for the blast model and previously published.[17a,20,41,44] We will evaluate three drug doses at the 7 dpi time point, but only evaluate the optimal dose at 28 dpi time point to minimize excessive animal usage. For the 7 dpi analysis, open field and NOR analysis will be performed, whereas Rotarod and Morris water maze analysis will be examined in the 28 dpi mice (n=15/group). Rotarod analysis will be tested at 3, 7, 14 and 28 dpi, while Morris water maze animals will be tested at 13 dpi, since pre-training is required for both behavioral assessments.

Expected Outcomes. We expect to identify at least one orally-active Sig2R/TMEM97 modulator with excellent ADME/PK properties that is neuroprotective and improves learning and memory in mice after brain injury in two distinct models. We anticipate that this candidate will be suitable for further for assessment of hERG trafficking, CYP induction, and full ADME-toxicology that will lead to its advancement as a clinical lead to treat TBI in humans by a new mechanism of action. Brains from animals (sham/vehicle, sham/drug, injured/vehicle, and injured/drug) from both injury models will be frozen for use in Aim 3.

Pitfalls and Alternatives. Based on our experience with the TBI models and proposed analyses for each laboratory, we do not anticipate any problems meeting our goals in this aim. We recognize that Sig2R/TMEM97 may also influence calcium homeostasis, synaptic functions, and inflammation, as well as Aβ and Tau production, but these experiments are beyond the scope of this proposal. Our focus on the core issues of tissue integrity, cell survival, neurogenesis and functional recovery are achievable within the 2-year time frame based on the personnel requested.

AIM 3. *Characterize binding of a Sig2R/TMEM97 modulator to receptors in the brain.*

Introduction. Although our compounds bind to Sig2R/TMEM97 in rat liver homogenates, it is critical to show that they bind to Sig2R/TMEM97 in the brain. Accordingly, the *objective* of this aim is to characterize the binding of a Sig2R/TMEM97 modulator in the mouse brain. Our *approach* will be to characterize how our lead Sig2R/TMEM97 modulator binds to different brain regions of animals (sham/vehicle, sham/compound, injured/vehicle, injured/compound) in blast and CCI injury models using quantitative autoradiography. The *rationale* for these studies is that we will learn how our lead candidate binds to Sig2R/TMEM97receptors in brains of uninjured and injured animals and whether treatment affects binding levels in those regions. The *positive impact* of these studies is that we will establish "target engagement" by showing that our compounds bind to Sig2R/TMEM97 in brain regions associated with cell survival and functional improvements.

Justification and Feasibility. Autoradiography studies using tritiated siramesine, a Sig2R/TMEM97 binding ligand, show a high density of Sig2R/TMEM97 in the motor cortex and hippocampus.[46] Sig2R/TMEM97 is also a marker for cell proliferation associated with cancer, and highly selective Sig2R/TMEM97 binding ligands have been used to image brain tumors.[47] Mach has shown that a Sig2R/TMEM97 fluorescent probe binds to cultured rat hippocampal and cortical neurons.[28] It has also been shown that Sig2R/TMEM97 binds Aβ oligomers in cultured neurons *in vitro*, and compounds that that block Aβ binding to Sig2R/TMEM97 are neuroprotective.[9] Importantly, these compounds, which also improve cognitive performance in Tg mice, were shown in autoradiography studies to bind to human anterior cortex tissue.

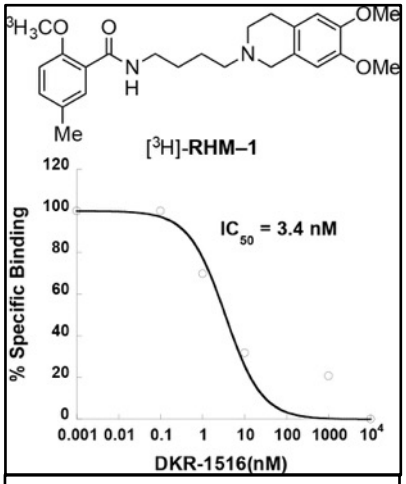


Fig. 13. Quantitative autoradiography competitive binding assay. **DKR-1516** displaces [^{3}H]**RHM-1** (10 nM) from Sig2R/TMEM97 in rat brain sections (IC_{50} = 3.4 nM); in triplicate.

We will collaborate with Jinbin Xu (Washington University in St. Louis; see supporting letter and biosketch), who has extensive experience in quantitative autoradiography and imaging of Sig2R/TMEM97 receptors in brains from humans, nonhuman primates, and rodents.[9,48] In a critical preliminary experiment to establish target engagement, we found in a competitive binding assay that unlabeled **DKR-1516** displaces [^{3}H]**RHM-1** (Fig. 13), a highly selective and high

affinity Sig2R/TMEM97 ligand, from Sig2R/TMEM97 in whole rat brain sections with an IC_{50} = 3.4 nM, an affinity that closely mirrors the observed affinity of **DKR-1516** in rat liver homogenate (K_i = 5.7 nM) (Fig. 13). This finding shows **DKR-1516** has high affinity for Sig2R/TMEM97 receptors in rodent brain, *which is 81% identical to the human receptor.* In a receptor occupancy study carried out with Robert Mach (University of Pennsylvania; see supporting letter and biosketch) using positron emission tomography (PET), we find that **DKR-1516** rapidly crosses the blood brain barrier and displaces the known Sig2R/TMEM97-selective ligand [^{11}C]**CTX** from receptors in the anterior cortex and other brain regions (not shown) of a rhesus monkey (Fig. 14). *These key preliminary studies establish target engagement and the feasibility of the proposed imaging experiments to characterize brain binding profiles in uninjured and injured mice (untreated and treated) using a radiolabeled analog of our lead Sig2R/TMEM97 modulator.*

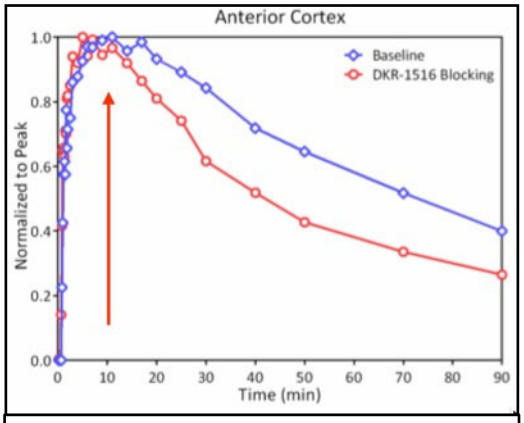


Fig. 14. Displacement of radiotracer [^{11}C]**CTX** (Sig2R/TMEM97, K_i=8.5 nM) with **DKR-1516** from receptors in anterior cortex of anesthetized rhesus monkey. **DKR-1516** (0.1 mg/kg) administered at 10 min post-injection of radiotracer (red arrow).

Research Design.

(1) Preparation of radioligands. A tritiated analog of the lead Sig2R/TMEM97 modulator used in Aim 2 (see *Research Design*, part 3*),* which is designated as [^{3}H]**STM-lead** for this discussion, will be custom synthesized by American Radiolabeled Chemicals (St. Louis, MO; see email) from a precursor we will provide (see Aim 1, *Research Design*, part 8) and used for systematic autoradiography studies. [^{3}H]**RHM-1**, a selective Sig2R/TMEM97 radioligand that has been well characterized by Xu's group,[49] will also be synthesized by American Radiolabeled Chemicals.

(2) Preparation of postmortem brain tissue sections. Brains will be collected from animals in the blast and CCI injury studies in Aim 2, fresh frozen using dry ice, and shipped to Xu's laboratory. Four different animal groups (n=6, sham/vehicle, sham/**STM-lead**, injured/vehicle, and injured/**STM-lead** at a single time point 7dpi; total of 48 animals) will be used for the autoradiography imaging studies. Serial coronal sections (20 μm thick) will be cut using a cryostat through the whole brain and stored at –80 °C until used for autoradiography.

(3) Competitive binding assay of unlabeled Sig2R/TMEM97 ligands with radioligands. Mouse brain sections will be incubated with [^{3}H]**RHM-1** and [^{3}H]**STM-lead** (single concentration ~K_d) and unlabeled Sig2R/TMEM97 ligands (**RHM-1**, siramesine, and **STM-lead**) at concentrations ranging from 0.1 nM to 10 μM will be added to acquire competition inhibition curves with [^{3}H]**RHM-1** and [^{3}H]**STM-lead**. Data from these experiments will be modeled using nonlinear regression analysis to determine the concentration of inhibitor that inhibits 50% of the specific binding of the radioligand (IC_{50} value). Competitive inhibition constants (K_i values) will be calculated from the IC_{50} values using the Cheng and Prusoff equation.[50]

(4) Determining the binding affinity and binding density using autoradiography. Slides containing adjacent coronal cryosections from mouse brain after blast and CCI injury and age and sex-matched sham control cohorts (n=48) will be incubated with [^{3}H]**RHM-1** and [^{3}H]**STM-lead** at a concentration of 0.1~20 nM and imaged using the Beta Imager. Quantitative analysis of the autoradiography will be done with the Beta-Image Plus software. Regions of interest will be outlined and results expressed as dpm/mm^2. A standard [^{3}H]Microscale slide obtained from American Radiolabeled Chemicals will be counted with the slides containing tissue for quantitative analysis of the radioactivity. Binding density will be measured for each concentration, and the dose-response curve will be generated and transformed into Scatchard plots. The corresponding dissociation constant (K_d) and binding density (B_{max}) in different brain regions (cortex, hippocampus, striatum, thalamus, cerebellum and brain stem) will be determined by a linear regression analysis of the transformed data according to formula (1).[51] Adjacent tissue sections will be stained with myelin marker and scanned using a NanoZoomer digital scanner to obtain the high quality bright field anatomical image to identify the regions of interest for autoradiography analysis.

$$\frac{B_s}{F} = -\frac{1}{K_d} B_s + \frac{1}{K_d} B_{max} \quad (1)$$

Expected Outcomes. We expect to show that **STM-lead** binds selectively to Sig2R/TMEM97 in rodent brains with high affinity and receptor density that are in agreement with the known Sig2R/TMEM97 binder [^{3}H]**RHM-1**. We will learn whether there are differences in Sig2R/TMEM97 binding and distribution in the different brain regions of animals after two types of TBI compared to their sham controls. We will also learn whether this Sig2R/TMEM97 modulator can restore Sig2R/TMEM97 density and homeostasis and reduce axonal and oligodendrocyte death in injured animals.

Pitfalls and Alternatives. Given our experience and preliminary work, we do not anticipate any problems. Future efforts will be directed toward using postmortem human tissues to conduct imaging studies to validate the Sig2R/TMEM97 engagement in humans suffering from acute or chronic TBI.

REFERENCES

1. Sivanandam, T. M.; Thakur, M. K. "Traumatic brain injury: a risk factor for Alzheimer's disease." *Neurosci. Biobehav. Rev*. **2012** *36*, 1376-1381.
2. (a) Faul, M.; Xu, L.; Wald, M. M.; Coronado, V. "Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths, 2002-2006." Atlanta, Georgia: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control; 2010. (b) http://www.ninds.nih.gov/disorders/tbi/detail_tbi.htm.
3. Saatman, K. E.; Duhaime, A.-C.; Bullock, R.; Maas, A. I. R.; Valadka, A.; Manley, G. T. Classification of Traumatic Brain Injury for Targeted Therapies. *J. Neurotrauma* **2008**, *25*, 719–738.
4. www.cognosci.com/documents/tbi_white_paper.pdf
5. For advantages of using multipotential therapeutic strategies to treat TBI, see: Cheng, G.; Kong, R.; Zhang, L.; Zhang, J. "Mitochondria in traumatic brain injury and mitochondrial-targeted multipotential therapeutic strategies." *Br. J. Pharmacol.* **2012**, *167*, 699–719.
6. (a) Hellewell, S. B.; Bowen, W. D. "A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain." *Brain Res.* **1990**, *527*, 244−253. (b) Hellewell, S. B.; Bruce, A.; Feinstein, G.; Orringer, J.; Williams, W.; Bowen, W. D. "Rat liver and kidney contain high densities of sigma-1 and sigma-2 receptors: characterization by ligand binding and photoaffinity labeling." *Eur. J. Pharmacol.* **1994**, *268*, 9–18.
7. (a) Mach, R. H.; Zheng, C.; Hawkins, W. G. "The σ2 Receptor: A Novel Protein for the Imaging and Treatment of Cancer." *J. Med. Chem.* **2013**, *56*, 7137-7160. (b) Huang, Y.S.; Lu, H.L.; Zhang, L.J.; Wu, Z. "Sigma-2 receptor ligands and their perspectives in cancer diagnosis and therapy." *Med. Res. Rev.* **2014**, *34*, 532-566. (c) Guo, L.; Zhen, X. "Sigma-2 receptor ligands: neurobiological effects." *Curr. Med. Chem.* **2015**, *22*, 989-1003.
8. Yi, B.; Sahn, J. J.; Ardestania, P. M.; Evans, A. K.; Scott, L.; Chan, J. Z.; Iyer, S.; Crisp, A.; Zuniga, G.; Pierce-Shimomura, J.; Martin, S. F.; Shamloo, M. *J. Neurochem.* **2017**, doi: 10.1111/jnc.13917. "Small molecule modulator of sigma 2 receptor is neuroprotective and reduces cognitive deficits and neuroinflammation in experimental models of Alzheimer's disease."
9. (a) Izzo, N. J.: Staniszewski, A.; To, L.; Fa, M.; Teich, A. F.; Saeed, F.; Wostein, H.; Walko, T, III.; Vaswani, V.; Wardius, M.; Syed, Z.; Ravenscroft, J.; Mozzoni, K.; Silky, C.; Rehak, C.; Yurko, R.; Finn, P.; Look, G.; Rishton, G.; Safferstein, H.; Miller, M.; Johanson, C.; Stopa, E.; Windisch, M.; Hutter-Paier, B.; Shamloo, M.; Arancio, O.; Levine, H, III, Catalano, S. M. "Alzheimer's Therapeutics Targeting Amyloid Beta 1–42 Oligomers I: Abeta 42 Oligomer Binding to Specific Neuronal Receptors Is Displaced by Drug Candidates That Improve Cognitive Deficits." *PLOS ONE* **2014**, *9,* e111898. (b) Izzo, N. J.; Xu, J.; Zeng, C.; Kirk, M. J.; Mozzoni, K.; Silky, C.; Rehak, C.; Yurko, R.; Look, G.; Rishton, G.; Safferstein, H.; Cruchaga, C.; Goate, A.; Cahill, M. A.; Arancio, O.; Mach, R. H.; Craven, R.; Head, E.; LeVine, H, III.; Spires-Jones, T. L.; Catalano, S. M. "Alzheimer's Therapeutics Targeting Amyloid Beta 1–42 Oligomers II: Sigma-2/PGRMC1 Receptors Mediate Abeta 42 Oligomer Binding and Synaptotoxicity." *PLOS ONE* **2014**, *9,* e111899.
10. Xu, J.; Zeng, C.; Chu, W.; Pan, F.; Rothfuss, J. M.; Zhang, F.; Tu, Z.; Zhou, D.; Zeng, D.; Vangveravong, S.; Johnston, F.; Spitzer, D.; Chang, K. C.; Hotchkiss, R. S.; Hawkins, W. G.; Wheeler, K. T.; Mach, R. H. "Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site." *Nat. Commun.* **2011**, 2:380.
11. Colabufo, N. A.; Berardi, F.; Abate, C.; Contino, M.; Niso, M.; Perrone, R. "Is the σ2 receptor a histone binding protein?" *J. Med. Chem.* **2006**, *49*, 4153−4158.
12. Alon, A.; Schmidt, H. R.; Wood, M. D.; Sahn, J. J.; Martin, S. F.; Kruse, A. C. "Molecular cloning of the σ_2 receptor." *Nature*, submitted.
13. (a) Wilcox, C. B.; Feddes, G. O.; Willett-Brozick, J. E.; Hsu, L. C.; DeLoia, J. A.; Baysal, B. E. "Coordinate up-regulation of TMEM97 and cholesterol biosynthesis genes in normal ovarian surface epithelial cells treated with progesterone: implications for pathogenesis of ovarian cancer." *BMC Cancer* **2007**, 7:223. (b) Bartz. F.; Kern, L.; Erz, D.; Zhu, M.; Gilbert, D.; Meinhof, T.; Wirkner ,U.; Erfle, H.; Muckenthaler, M.; Pepperkok, R.; Runz, H. "Identification of cholesterol-regulating genes by targeted RNAi screening." *Cell Metab.* **2009**, *10*, 63-75. (c) Richardson, K.; Ordovas, J. M. "Functional genomic and epidemiological studies reveal novel genes regulating cholesterol metabolism." *Genome Med.* **2009**, *1*, 96. (d) Sanchez-Pulido, L.; Ponting, C. P. "TM6SF2 and MAC30, new enzyme homologs in sterol metabolism and common metabolic disease." *Front. Genet*. **2014**, *5*:439.

14. (a) Xu, X.-Y.; Zhang, L.-J.; Yu,Y.-Q.; Zhang, X.-T.; Huang, W.; Nie, X.-C.; Song, G.-Q. "Down-regulated MAC30 expression inhibits proliferation and mobility of human gastric cancer cells." *Cell. Physiol. Biochem.* **2014**, *33*, 1359-1368. (b) Qiu, G.; Sun, W.; Zou, Y.; Cai, Z.; Wang, P.; Lin, X.; Huang, J.; Jiang, L.; Ding, X.; Hu, G. "RNA interference against TMEM97 inhibits cell proliferation, migration, and invasion in glioma cells." *Tumour Biol.* **2015**, *36*, 8231–8238.
15. See: (a) http://mouse.brain-map.org/experiment/show?id=68666511. (b) http://www.proteinatlas.org/ENSG00000109084-TMEM97/tissue.
16. (a) Schouten, J. W. "Neuroprotection in traumatic brain injury: a complex struggle against the biology of nature." *Curr. Opin. Crit. Care* **2007**, *13,* 134–142. (b) Loane, D. J.; Faden, A. I. Neuroprotection for Traumatic Brain Injury: Translational Challenges and Emerging Therapeutic Strategies. *Trends Pharmacol. Sci.* **2010**, *31*, 596–604. (c) Kumar, A.; Loane, D. J. "Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention." *Brain Behav. Immun*. **2012**, *26*, 1191-1201. (d) Kabadi, S. V.; Faden, A. I. "Neuroprotective Strategies for Traumatic Brain Injury: Improving Clinical Translation." *Int. J. Mol. Sci.* **2014***, 15*, 1216-1236. (e) Lozano, D.; Gonzales-Portillo, G. S.; Acosta, S.; de la Pena, I.; Tajiri, N.; Kaneko, Y.; Borlongan, C. V. "Neuroinflammatory responses to traumatic brain injury: etiology, clinical consequences, and therapeutic opportunities." *Neuropsychiatr. Dis. Treat.* **2015**, *11*, 97–106. (f) Bergold, P. J. "Treatment of traumatic brain injury with anti-inflammatory drugs." *Exp. Neurol.* **2016**, *275*, 367-380. (g) Russo, M. V.; McGavern, D. B. "Inflammatory neuroprotection following traumatic brain injury." *Science*, **2016**, *353*, 783-785.
17. (a) Yin, T. C.; Britt, J. K.; De Jesus-Cortes, H.; Lu, Y.; Genova, R. M.; Khan, M. Z.; Voorhees, J. R.; Shao, J.; Katzman, A. C.; Huntington, P. J.; Wassink, C.; McDaniel, L.; Newell, E. A.; Dutca, L. M.; Naidoo, J.; Cui, H.; Bassuk, A. G.; Harper, M. M.; McKnight, S . L.; Ready, J. M.; Pieper, A. A. "P7C3 neuroprotective chemicals block axonal degeneration and preserve function after traumatic brain injury." *Cell Reports* **2014**, *8*, 1731–1740. (b) Blaya, M.O.; Bramlett, H. M.; Naidoo, J.; Pieper, A. A.; Dietrich, W. D. "Neuroprotective efficacy of a proneurogenic compound after traumatic brain injury." *J. Neurotrauma* **2014**, *31*, 476-486. (c) Wang, G.; Han, T.; Nijhawan, D.; Theodoropoulos, P; Naidoo, J.; Yadavalli, S.; Mirzaei, H.; Pieper, A. A.; Ready, J. M.; McKnight, S. L. "The P7C3 class of neuroprotective chemicals functions by activating the rate-limiting enzyme in NAD salvage." *Cell* **2014**, *158*, 1324-1334.
18. (a) Laskowitz, D. T.; McKenna, S. E.; Wang, H.; Durham, L.; Yeung, N.; Christensen, D.; Vitek, M. P. "COG1410, a novel apolipoprotein E-based peptide, improves functional recovery in a murine model of traumatic brain injury." *J. Neurotrauma* **2007**, *24,* 1093-1107. (b) Kaufman, N. A.; Beare, J. E.; Tan, A. A.; Vitek, M. P.; McKenna, S. E.; Hoane, M. R. "COG1410, an Apolipoprotein E-based Peptide, Improves Cognitive Performance and Reduces Cortical Loss Following Moderate Fluid Percussion Injury in the Rat." *Behav. Brain Res.* **2010,** *214,* 395-401.
19. (a) Zheng, W.; Watts, L. T.; Holstein, D. M.; Prajapati, S. I.; Keller, C.; Grass, E. H.; Walter, C. A.; Lechleiter, J. D. "Purinergic receptor stimulation reduces cytotoxic edema and brain infarcts in mouse induced by photothrombosis by energizing glial mitochondria." *PLoS One* **2010**, *5*, e14401. (b) Weisman, G. A.; Woods, L. T.; Erb, L.; Seye, C. I. "P2Y Receptors in the Mammalian Nervous System: Pharmacology, Ligands and Therapeutic Potential." *CNS Neurol .Disord. Drug Targets* 2012, *11*, 722–738.
20. Dixon, K.J.; Theus, M. H.; Nelersa, C. M.; Mier, J.; Travieso, L. G.; Yu, T. S.; Kernie, S. G.; Liebl, D. J. "Endogenous neural stem/progenitor cells stabilize the cortical microenvironment after traumatic brain injury." *J. Neurotrauma* **2015**, *32*, 753-764.
21. For a recent review, see: Takeuchi, T.; Duszkiewicz, A. J.; Morris, R. G. M. "The synaptic plasticity and memory hypothesis: encoding, storage and persistence." *Philos. Trans. R. Soc. B: Biol. Sci.* **2014**, *36* (1633): 20130288.
22. Osler, N. D.; Carlson, S. W.; DeSana, A.; Dixon, C. E. "Chronic histopathological and behavioral outcomes of experimental traumatic brain injury in adult male animals." *J. Neurotrauma* **2015**, *32*, 1861-1882.
23. (a) Sunderhaus, J. D.; Dockendorff, C.; Martin, S. F. "Synthesis of Diverse Heterocyclic Scaffolds via Tandem Additions to Imine Derivatives and Ring-Forming Reactions." *Tetrahedron* **2009**, *65*, 6454-6469. (b) Martin, S. F. "Strategies for the Synthesis of Alkaloids and Novel Nitrogen Heterocycles." In *Adv. Heterocyclic Chem.* 2013, **110**, 73-117. (c) Sahn, J. J.; Granger, B. A.; Martin, S. F. "Evolution of a Strategy for Preparing Bioactive Small Molecules by Sequential Multicomponent Assembly Processes, Cyclizations, and Diversification." *Org. Biomol. Chem.* **2014**, *12*, 7659-7672.
24. (a) Sahn, J. J.; Su, J.; Martin, S. F. “Facile and unified approach to skeletally diverse, privileged scaffolds.” *Org. Lett.* **2011**, *13*, 2590. (b) Sahn, J. J.; Martin, S. F. “Facile syntheses of substituted, conformationally-constrained benzoxazocines and benzazocines via sequential multicomponent assembly and cy-

clization." *Tetrahedron Lett.* **2011**, *52*, 6855-6858. (c) Sahn, J. J.; Martin, S. F "Expedient synthesis of norbenzomorphan library via multicomponent assembly process coupled with ring-closing reactions." *ACS Combi. Sci.* **2012**, *14*, 496-502

25. Sahn, J. J.; Hodges, T.; Chan, J.; Martin, S. F. "Norbenzomorphan Framework as a Novel Scaffold for Generating Sigma 2 Subtype Selective Ligands." *ChemMedChem* doi: 10.1002/cmdc.201500551.
26. Banister, S. D.; Kassioiu, M. "The Therapeutic Potential of Sigma (σ) Receptors for the Treatment of Central Nervous System Diseases: Evaluation of the Evidence." *Curr. Pharm. Des.* **2012**, *18,* 884-901.
27. (a) Crisp, A., Pierce-Shimomura, J.T. (2012) Induction and prevention of patterned neurodegeneration by amyloid precursor protein. Society for Neuroscience, New Orleans, LA, Abstract # 343.20. (b) Crisp, A.; Iyer, S.; Pierce-Shimomura, J.T. "Induction and prevention of patterened neurodegeneration by Amyloid Precursor Protein." *in preparation*.
28. Zeng, C.; Garg, N.; Mach, R.H. "The PGRMC1 Protein Level Correlates with the Binding Activity of a Sigma-2 Fluorescent Probe (SW120) in Rat Brain Cells." *Mol. Imaging Biol.* **2016**, *18*, 172-179.
29. Guennoun, R.; Meffre, D.; Labombarda, F.; Gonzalez, S. L.; Gonzalez Deniselle, M. C.; Stein, D. G.; De Nicola, A. F.; Schumacher, M. "The membrane associated progesterone-binding protein 25-Dx: expression, cellular localization and up-regulation after brain and spinal cord injuries." *Brain Res. Rev.* **2008**, *57*, 493–505.
30. For leading references of PGRMC1, see: (a) Cahill, M. A. "Progesterone receptor membrane component 1: an integrative review." *J. Steroid Biochem. Mol. Biol.* **2007**, *105*, 16-36. (b) Rohe, H. J.; Ahmed, I. S.; Twist, K. E.; Craven, R. J. "PGRMC1 (progesterone receptor membrane component 1): a targetable protein with multiple functions in steroid signaling, P450 activation and drug binding." *Pharmacol. Ther.* **2009**, *121*, 14-19. (c) Thomas, P.; Pang, Y. "Membrane Progesterone Receptors: Evidence for Neuroprotective, Neurosteroid Signaling and Neuroendocrine Functions in Neuronal Cells." *Neuroendrocrinology* **2012**, *96*, 162-171. (d) Kabe, Y.; Nakane, T.; Koike, I.; Yamamoto, T.; Sugiura, Y.; Harada, E.; Sugase, K.; Shimamura, T.; Ohmura, M.; Muraoka, K.; Yamamoto, A.; Uchida, T.; Iwata, S.; Yamaguchi, Y.; Krayukhina, E.; Noda, M.; Handa, H.; Ishimori, K.; Uchiyama, S.; Kobayashi, T.; Suematsu, M. "Haem-dependent dimerization of PGRMC1/Sigma-2 receptor facilitates cancer proliferation and chemoresistance." *Nature Comm.* **2016**, *7*:11030. (e) Cahill, M. A.; Jazayeri, J. A.; Catalano, S. M.; Toyokuni, S.; Kovacevic, Z.; Richardson, D. R. "The emerging role of progesterone receptor membrane component (PGRMC1) in cancer biology." *Biochim. Biophys. Acta (BBA)-Reviews on Cancer*. http://dx.doi.org/10.1016/j.bbcan.2016.07.004.
31. Laurini, E.; Zempieri, D.; Mamolo, M. G.; Vio, L.; Zanette, C.; Florio, C.; Posocco, P.; Fermeglia, M.; Pricl, S. "A 3D-pharmacophore model for σ_2 receptors based on a series of substituted benzo[d]oxazol-2(3*H*)-one derivatives." *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2954-2957.
32. Zeng, C.; Rothfuss, J. M.; Zhang, J.; Vangveravong, S.; Chu, W.; Li, S.; Tu. Z.; Xu, J.; Mach, R. H. " Functional assays to define agonists and antagonists of the sigma-2 receptor." *Anal. Biochem.* **2014**, *448*, 68-74.
33. (a) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings." *Adv. Drug Delivery Rev.* **2001**, *46*, 3−26. (b) Gleeson, M. P. "Generation of a set of simple, interpretable ADMET rules of thumb." *J. Med. Chem*. **2008**, *51*, 817-834. (c) Kerns, E. H; Di, L. "Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization." Academic Press, Boston, 2008.
34. (a) Kelder, J.; Grootenhuis, P.D.J.; Bayada, D.M.; Delbressine, L. P. C.; Ploemen, J-P. "Polar Molecular Surface as a Dominating Determinant for Oral Absorption and Brain Penetration of Drugs." *Pharm. Res.* **1999**, *16*, 1514-1519. (b) Wager, T. T.; Chandrasekaran, R. Y.; Hou, X.; Troutman, M. D.; Verhoest, P. R.; Villalobos, A.; Will, Y. "Defining desirable central nervous system drug space through the alignment of molecular properties, in vitro ADME, and safety attributes." *ACS Chem. Neurosci.* **2010,** *1*, 420-34.
35. For example, see: Cumming, J. G.; Bower, J. F.; Waterson, D.; Faull, A.; Poyser, P. J.; Turner, McDermott, P. B.; Campbell, A. D.; Hudson, J.; James, M.; Winter, J.; Wood, C. "The design and synthesis of novel, potent and orally bioavailable N-aryl piperazine-1-carboxamide CCR2 antagonists with very high hERG selectivity." *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3895-3899.
36. Muller, K.; Bohm, H-. J. "Facilitating the design of fluorinated drugs." *Chem. Biol.* **2009**, *16*, 1130-1131.
37. PDSP assay protocol book accessible at https://pdspdb.unc.edu/pdspWeb/?site=binding

38. Bowes, J.; Brown, A. J.; Hamon, J.; Jarolimek, W.; Sridhar, A.; Waldron, G.; Whitebread, S. "Reducing safety-related drug attrition: the use of *in vitro* pharmacological profiling." *Nature Rev. Drug Discovery* **2012**, *11*, 909-922.
39. Feng, B.; Mills, J. B.; Davidson, R. E.; Mireles, R. J.; Janiszewski, J. S.; Troutman, M. D.; de Morais, S. M. "In Vitro P-glycoprotein Assays to Predict the in Vivo Interactions of P-glycoprotein with Drugs in the Central Nervous System." *Drug Metab. Dispos.* **2008**, *36*, 268-275.
40. (a) Goldstein, L. E.; Fisher, A. M.; Tagge, C. A. et al. "Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model." *Sci. Transl. Med.* **2012**, 4(134), 134ra60. (b) Mohan, K.; Kecova, H.; Hernandez-Merino, E.; Kardon, R. H.; Harper, M. M. *"Retinal ganglion cell damage in an experimental rodent model of blast-mediated traumatic brain injury."* Invest. Ophthalmol. Vis. Sci. **2013**, *54*, 3440-3450.
41. Theus, M. H.; Ricard, J.; Glass, S. J.; Travieso, L. G.; Liebl, D. J. "EphrinB3 blocks EphB3 dependence receptor functions to prevent cell death following traumatic brain injury." *Cell Death Dis.* **2014**, *5*, e1207.
42. Brodziak, A.; Kolat, E.; Rozyk-Myrta, A. "In search of memory tests equivalent for experiments on animals and humans." *Med. Sci. Monit.* **2014**, *20*, 2733-2739.
43. Northington, F. J.; Ferriero, D. M.; Graham, E. M.; Traystman, R. J.; Martin, L. J. "Early neurodegeneration after hypoxia-ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis." *Neurobiol. Dis*. **2001**, *8*, 207–219.
44. (a) Theus M, Ricard J, Bethea J, and Liebl D. "EphB3 inhibits the expansion of neural progenitor cells in the SVZ by regulating p53 during homeostasis andfollowing traumatic brain injury." *Stem Cells* **2010**, 28, 1231-1242. (b) Perez E, Cepero M, Perez S, Coyle J, SIck T, and Liebl D. "EphB3 signaling propagates synaptic dysfunction in the traumatic injured brain." *Neurobiol. Dis.* **2016**, 94, 73-84.
45. (a) Ricard, J.; Salinas, J.; Garcia, L.; Liebl, D. J. "EphrinB3 regulates cell proliferation and survival in adult neurogenesis." *Mol. Cell Neurosci.* **2006**, *31*, 713-722. (b) Yu, T. S.; Zhang, G.; Liebl, D. J.; Kernie, S. G. "Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors." *J. Neurosci.* **2008**, *28*, 12901-12912.
46. Søby, K. K.; Mikkelsen, J. D.; Meier, E.; Thomsen, C. "Lu 28-179 labels a sigma-2 site in rat and human brain." *Neuropharmacol.* **2002**, *43*, 95−100.
47. Tu, Z.; Xu, J.; Jones, L. A.; Li, S.; Dumstorff, C.; Vangveravong, S.; Chen, D. L.; Wheeler, K. T.; Welch, M. J.; Mach, R. H. "Fluorine-18-labeled benzamide analogues for imaging the sigma-2 receptor status of solid tumors with positron emission tomography." *J. Med. Chem.* **2007**, *50*, 3194−3204.
48. (a) Xu, J.; Chu, W.; Tu, Z.; Jones, L. A.; Luedtke, R. R.; Perlmutter, J. S. *et al*. "[(3)H]4-(Dimethylamino)-N-[4-(4-(2-methoxyphenyl)piperazin- 1-yl)butyl]benzamide, a selective radioligand for dopamine D(3) receptors. I. In vitro characterization." *Synapse* **2009**, *63*, 717-728. (b) Xu, J.; Hassanzadeh, B.; Chu, W.; Tu, Z,; Jones, L. A.; Luedtke, R. R. *et al*. "[3H]4-(dimethylamino)-N-(4-(4-(2-methoxyphenyl)piperazin-1-yl) butyl)benzamide: a selective radioligand for dopamine D(3) receptors. II. Quantitative analysis of dopamine D(3) and D(2) receptor density ratio in the caudate-putamen." *Synapse* **2010**, *64*, 449-459. (c) Sun, J.; Xu, J.; Cairns, N. J.; Perlmutter, J. S.; Mach, R. H. "Dopamine D1, D2, D3 receptors, vesicular monoamine transporter type-2 (VMAT2) and dopamine transporter (DAT) densities in aged human brain." *PloS One*, **2012**, e49483. (d) Sun, J.; Cairns, N. J.; Perlmutter, J. S.; Mach, R. H.; Xu, J. "Regulation of dopamine D3 receptor in the striatal regions and substantia nigra in diffuse Lewy body disease." *Neurosci*. **2013**, *248*, 112-126. (e) Sun, J.; Kouranova, E.; Cui, X.; Mach, R. H.; Xu, J. "Regulation of dopamine presynaptic markers and receptors in the striatum of DJ-1 and Pink1 knockout rats." *Neurosci. Lett.* **2013** *557*, Pt B, 123-128.
49. Xu, J.; Tu, Z.; Jones, L. A.; Vangveravong, S.; Wheeler, K. T.; Mach, R. H. "[^{3}H]N-[4-(3,4-dihydro-6,7-dimethoxyisoquinolin-2(1H)-yl)butyl]-2-methoxy-5-methylbenzamide: a novel sigma-2 receptor probe." *Eur. J. Pharmacol.* **2005**, *525*, 8-17. (PMID:16289030)
50. Cheng, Y.; Prusoff, W. H. "Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction." *Biochem. Pharmacol*. **1973**, *22*, 3099-3108.
51. Scatchard, G. "The attractions of proteins for small molecules and ions." *Ann NY Acad Sci*. **1949**, *51*, 662-672.