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In Silico Modelling of Novel Drug Ligands Associated with Abnormal Tau Phosphorylation: Implications for Concussion Associated Tauopathy Intervention[†]

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Abstract

The objective of this study was to develop an *in silico* screening model for characterization of potential novel ligands from commercial drug libraries able to functionally activate certain olfactory receptors (ORs), which are members of the class A rhodopsin-like family of G protein couple receptors (GPCRs), in the brain of murine models of concussion. We previously found that concussions may significantly influence expression of certain ORs, e.g. OR4M1 in subjects with a history of concussion/traumatic brain injury (TBI). In this study we built a 3-D OR4M1 model and used it in *in silico* screening of potential novel ligands from commercial drug libraries. We report that *in vitro* activation of OR4M1 with the commercially available ZINC library compound 10915775 led to a significant attenuation of abnormal tau phosphorylation in embryonic cortico-hippocampal neuronal cultures derived from NSE-OR4M1 transgenic mice, possibly through modulation of the JNK signaling pathway. The attenuation of abnormal tau phosphorylation was rather selective since ZINC10915775 significantly decreased tau phosphorylation on tau Ser202/T205 (AT8 epitope) and tau Thr212/Ser214 (AT100 epitope), but not on tau Ser396/404 (PHF-1 epitope). Moreover, no response of ZINC10915775 was found in control hippocampal neuronal cultures derived from wild type littermates. Our *in silico* model provides novel means to

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Conflict Of Interest

The authors declare no conflict of interest.

pharmacologically modulate select ubiquitously expressed ORs in the brain through high affinity ligand activation to prevent and eventually to treat concussion induced down regulation of ORs and subsequent cascade of tau pathology.

Keywords

concussion; olfactory receptor; G protein-coupled receptors; in silico screening; tau phosphorylation

Introduction

Emerging synthetic biology technologies have transformed the way systems biologists and pharmacologists deconstruct the involvement of G protein-coupled receptors (GPCRs) in a variety of physiological and translational settings (Urban & Roth 2015). For example we previously reported altered expression of the olfactory receptors (OR) members of the class A rhodopsin-like family of GPCRs in subjects with history of concussion (Zhao et al. 2013). Most interestingly, we previously found an association between the content of abnormally phosphorylated microtubule associate protein, tau, and decreased expression of certain ubiquitously expressed ORs in the brain and in circulating mononuclear cells (Zhao et al. 2013).

While ORs have been largely investigated for their role in olfaction, little is known about ubiquitously distributed ORs in the brain. For example, it is well known that specific odorant ligands, when bound to select ORs in the olfactory mucosa, may transduce the sensation through OR-type G proteins consistent with the general concept that GPCRs, like olfactory ORs are membrane-bound proteins that are responsible for cell-environment communications. However, GPCRs, like other membrane-embedded proteins, have characteristics that make their 3-D structure difficult to determine experimentally. While the structures of several GPCRs have been determined recently, no experimental structures of ubiquitously expressed ORs are currently available.

The current study is designed to identify ligands for the ubiquitously expressed GPCR OR4M1 via in silico screening using a homologous model of OR. In particular we report for the first time the building of a novel 3-D OR model which we used in *in silico* screening of potential novel ligands from commercial drug libraries able to functionally activate OR signaling. This study provides the much needed information to potentially intervene pharmacologically to attenuate abnormal tau in the brain, a canonical neuropathological feature in response to concussions.

Materials and Methods

Homology Modeling and Binding Site Identification

A homology model of the GPCR OR4M1 was built using MODELLER version 9.12 (Eswar et al. 2003) using bovine rhodopsin as a template. Because the sequence similarity between OR4M1 and bovine rhodopsin is below 25% identity, the alignment of the two sequences was built using profiles of homologous olfactory receptors and other GPCRs. The profiles

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were matched to each other using the SALIGN routine of program MODELLER (Marti-Renom, Madhusudhan, & Sali 2004). The alignment was manually edited using predictions of transmembrane helices for OR4M1. The quality of the alignment was evaluated by mapping predicted olfactory receptor binding site residues (Man, Gilad, & Lancet 2004) onto the model. A divergent loop (Ala175-Pro175) was remodeled using the loop modeling routine of MODELLER (Fiser, Do, & Sali 2000). A total of 100,000 conformations for the loop were generated and the best scoring one, based on the DOPE potential (Shen & Sali 2006), was selected as the final model. The ligand-binding pocket in the final model was defined by the known OR binding site residues and by the SiteHound binding site identification program (Ghera & Sanchez 2009).

Docking, Scoring and Selection

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The pocket identified in the OR4M1 model was used as a target for virtual screening of small molecular compounds. A set of around five million lead-like compounds (Teague et al. 1999) derived from the ZINC library of commercially available compounds (Irwin & Shoichet 2005) was docked into the binding site using program DOCK version 6.5 (Moustakas et al. 2006). Lead-like compounds were selected to facilitate optimization of validated hits. Compounds for experimental testing were selected based on docking score and visual inspection of binding site complementarity and hydrogen bonding. Clustering of compounds based on chemical fingerprint similarity was carried out using single-linkage clustering and using a Tanimoto coefficient of 0.7 as the cutoff. Chemical fingerprint similarity was computed using the JChem software (ChemAxon).

Experimental Animals

Rat neuron-specific enolase (NSE) promoter plasmid containing human OR4M1 was constructed by inserting ~1kb cDNA fragment with the entire coding region of hOR4M1 (NM_001005500.1, OriGene Technologies, Inc. Rockville, MD) in the NotI site of the plasmid vector. A cassette of ~6 kb SalI fragment containing NSE promoter and hPGC-1 α was gel purified and microinjected into one-cell mouse egg (C57BL6 $\times$ SJL) as described previously (Kelley et al. 1999; Qin et al. 2006). TgOR4M1 founders were identified by PCR-based genotyping. All animals were maintained on a 12:12-h light/dark cycle with lights on at 07:00 h in a temperature-controlled ($20 \pm 2^\circ\text{C}$) vivarium, and all procedures were approved by the MSSM IACUC.

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The experimental concussion rodent model was generated by blast exposure, as previously described (Chavko, Prusaczyk, & McCarron 2006). Briefly, adult male Long Evans hooded rats (250–350g; 10–12 weeks of age) were used as subjects. Rats were exposed to overpressure injury using the Walter Reed Army Institute of Research (WRAIR) shock tube, which simulates the effects of air blast exposure under experimental conditions (Elder et al. 2012). Blood samples were withdrawn through the saphenous vein 1, 3, 6, and 12 months following the blast. At the end of the study, the cortex, hippocampus and cerebellum were collected and stored at -80°C until further analysis.

Primary neuron preparation, treatment and cAMP assay

Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from TgOR4M1 mice. Neurons were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5mM L-glutamine, and 1% penicillin-streptomycin (all from Life Technologies). On Day 5 of the culture, primary cortico-hippocampal neuron cultures were pretreated with 3-isobutyl-1-methylxanthine (IBMX, from Sigma-Aldrich), an inhibitor of cAMP phosphodiesterase, for 10 min followed by ligand treatment (10 μ M, 10 min) according to the manufacturer's instruction and consistent with previous evidence showing cAMP accumulation in a linear range fashion in HEK cells (Ferrandon et al. 2009) as assessed by colorimetric cAMP ELISA assay (Cell Biolabs). Ligands from the ZINC library were purchased from Enamine (<http://zinc.docking.org/substance/10915775>).

RNA extraction, cDNA synthesis and quantitative PCR

Rat blood RNA was extracted using the Ribopure RNA Purification kit (Life Technologies). One μ g of total RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Life Technologies). Quantitative PCR (qPCR) was performed in four replicates in the 7900HT Fast Real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primers used in qPCR assay are summarized in Table I. Fold changes were calculated using the 2^{-C_t} method as previously described (Zhao et al. 2013).

Assessment of tau phosphorylation

Twenty μ g protein lysate of either primary neuron or rat hippocampus samples were used for SDS-PAGE protein separation. Separated proteins were transferred onto nitrocellulose membrane, and western blotting was performed using 5% milk in Tris-buffered saline solution for blocking nonspecific binding sites and antibody dilutions. Primary antibodies against tau proteins included mouse monoclonal antibodies as follows (location of epitopes refers to the longest tau isoform of 441 amino acid residues): AT8 (pSer202/205), AT100 (pThr212/Ser214) and PHF-1 (pSer396/404). For assessment of active JNK, anti-phospho-JNK (Cell Signaling) was used as probe and β -actin signaling (Santa Cruz Biotechnology) was used controlled for samples loading. Antibodies were incubated for 1hr as previously described (Zhao et al. 2003).

Statistics

In these studies, all values are expressed as mean and standard error of the mean (SEM). Differences between means were analyzed using one-way ANOVA or two-tailed student t-test. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using the Prism Stat program (GraphPad Software, Inc.).

Results

Modeling of OR4M1

A model of the 3-D structure of olfactory receptor OR4M1 was built by comparative modeling based on the crystal structure of bovine rhodopsin (Okada et al. 2004). Most of the predicted binding site residues are spatially close in the model suggesting that the alignment is reliable. The alignment shows that the region between Ala 142 and Pro 175 of OR4M1 is highly divergent from the equivalent region in rhodopsin, hence this region was further refined using via loop modeling. In the final model most of the predicted binding site residues from Man, Gilad, & Lancet 2004 cluster into one region (Fig. I) which is consistent with a binding pocket identified in the model using program SiteHound (Gherzi & Sanchez 2009).

Docking and in silico screening

The pocket identified in the OR4M1 model was used as a target for virtual screening of a set of ~ five million lead-like commercially-available compounds (Irwin & Shoichet 2005). The top 700 hits from the screening (ranked by docking score) were visually inspected, and 57 compounds, which showed good occupancy of the predicted binding pocket and at least two hydrogen bonds with the protein, were selected, shown as a space filling model (Fig. IIA) and with predicted binding pocket (red mesh) (Fig. IIB). The selected compounds were clustered based on chemical similarity. This resulted in 32 clusters of structurally similar compounds. Based on the availability of these compounds, we chose 25 commercially available compounds that could be further grouped into 16 clusters of structurally similar compounds (Table II) for experimental validation.

In vitro validation of candidate OR4M1 ligands

In this study, embryonic cortico-hippocampal neuronal cultures from Tg NSE-OR4M1 mice were used as test candidates' compound to activate OR4M1. The intracellular signaling pathways activated by GPCR signaling include the cAMP/JNK pathway among others. Since OR4M1 belongs to the GPCR family, we treated TgOR4M1 neuronal cultures with individual ZINC ligands at 10 μ M concentration and assessed for OR4M1-mediated cellular induction of cAMP as an index of OR4M1 activation.

We found that among the 25 compounds tested, ZINC10915775 was particularly efficient in the activation of OR4M1 as indicated by increases in cellular cAMP contents in response to ligand treatment (24 hr) (Table II). These ring structures occupied the two wider regions in the OR4M1 pocket, while the linker occupied the narrow connecting region (Fig. III A, B).

Based on this evidence, we continued to test the dose-responsiveness of activation of OR4M1 by ZINC10915775 in E15 cortico-hippocampal cultures generated from OR4M1 transgenic mice. Using the cAMP assay, we found that the activation of OR4M1 by ZINC10915775 reached peak activity at 100 nM (Fig. IIIC), so we chose this concentration for further *in vitro* mechanistic studies.

OR4M1 ligand ZINC10915775 inhibited tau phosphorylation through inhibition of c-Jun N-terminal kinase (JNK) signaling

Based on this evidence we continued to explore whether OR4M1 activation could influence abnormal tau processing. In this study cortico-hippocampal neuron cultures derived from either TgOR4M1 mice or WT littermates ZINC10915775 (100 nM for 10 min). We found that treatment of TgOR4M1 neurons with the ZINC10915775 compound significantly decreased tau phosphorylation on Ser202/T205 (AT8 epitope) and Thr212/Ser214 (AT100 epitope), but not on Ser396/404 (PHF-1 epitope) (Fig. IVA).

Interestingly, we also found that attenuation of abnormal tau phosphorylation in the TgOR4M1 corticohippocampal neuron cultures in response to ZINC10915775 treatment coincided with a significant decrease in the levels of phosphorylated JNK (Fig. IVA).

However, no detectable change in tau phosphorylation or JNK was detected in WT neuron cultures upon ZINC10915775 treatment (Fig. IVB), suggesting that ZINC10915775-mediated activation of OR4M1 could lead to decreased tau phosphorylation, possibly through the inhibition of the JNK signaling pathway.

Expression of OR and tau phosphorylation in a rat model of blast-induced concussion

To examine whether the down-regulation of OR4M1 as well as the expression of three other ubiquitously expressed OR previously found decreased in subjects with histories of concussions (Zhao et al. 2013) we explored the expression of the rodent OR orthologs in an *in vivo* rat model of concussion for potential future *in silico* drug screening studies.

Excitingly, we found that similar to what was found in subjects with a history of concussion, the expression of Orl1612, Orl1671, Orl322 and Orl735 which are the rodent orthologs of the human, OR4M1 OR2J3, OR4D9 and OR4X1, respectively, showed a time-dependent decrease in PBMCs at 1, 3, 6, and 12 months in the same rat group post-blast-induced concussion compared to naïve control rats (Fig. V).

Discussion

Concussion is a form of mild traumatic brain injury (mTBI) and can occur after an impact to the head or after a whiplash-type injury that causes the head and brain to shake quickly back and forth (McCrory et al. 2005). Concussions are usually not life-threatening, but they can cause serious symptoms that require medical treatment. A concussion is a traumatic injury that might result in an altered mental state including unconsciousness (McCrory et al. 2005). For this reason concussions have received wide-spread attention especially for their potential implications from physical impacts in sports such as football or boxing where the risk to incur concussions increases exponentially. Interestingly, the Centers for Disease Control and Prevention (CDC) estimated that in 2010 approximately 2.5 million people in the US have experienced concussions which required medical assistance (Faul et al. 2010).

The current study is based on our recent observation that abnormal tau processing in subjects with a history of concussion may be traced back to abnormal down-regulation of certain ORs, including OR4M1 and OR11H1, in the brain (Zhao et al. 2013). In this study, we

demonstrated that activation of OR4M1 with select ligands led to reduction of phosphorylation at Ser202/205 and Thr212/Ser214 of tau and found that the reduction of tau phosphorylation was associated with altered activation of the JNK pathway. Interestingly the physiological relevance of OR4M1 is consistent with the recent study demonstrating that controlled cortical concussion activates JNK and increases tau phosphorylation in a 3xTg mouse model of AD (Tran, Sanchez, & Brody 2012).

In previous studies, we have found that activation of OR4M1 by acetophenone, a potential non-selective OR ligand which was not identified in the current *in silico* screening, failed to reveal attenuation in tau phosphorylation at the pS396/S404 epitope (Zhao et al. 2013). Indeed, the attenuation of abnormal tau phosphorylation we found in this *in silico* drug discovery study was rather selective since ZINC10915775 significantly decreased tau phosphorylation on tau Ser202/T205 (AT8 epitope) and tau Thr212/Ser214 (AT100 epitope) through activation of OR4M1, but not on tau Ser396/404 (PHF-1 epitope). This could possibly be explained by several factors. For example, it is possible that certain OR agonists might activate various signaling pathways differentially. If the differences in activation among OR agonists are large enough on one signaling pathway, a special phenomenon could be observed, i.e., only selective agonists can activate this signaling pathway, as we found in this study and summarized in Fig. VI. However, if the differences are large enough on two signaling pathways, agonist-selective activation on two pathways will occur, i.e., a group of agonists only activates one signaling pathway, while another group of agonists could selectively activate another signaling pathway. We infer that the agonist-selective signaling ZINC10915775 observed in this study can be considered as the “selection between signaling pathways” or “pathway-selective signaling” as previously discussed (Zheng, Loh, & Law 2010). More studies aimed at the characterization of additional putative OR4M1 ligands will clarify this important issue. To explore the cause-effect relationship between OR down-regulation and concussion-type neuropathology, we used a blast-induced concussion rat model. Consistent with our observation in human concussion subjects (Zhao et al. 2013), we found significantly lower contents of OR4M1, and other select ORs previously found in the blood of blast-injured rats compared to control rats over 12 months following concussion.

Our studies have identified a novel ligand for OR4M1 concussion biomarker through *in silico* screening, and activation of OR4M1 by this ligand in primary neuronal cultures resulted in decreased tau phosphorylation through inhibition of the JNK signaling pathway. Taking these findings together, our hypothesis is that inactivation of certain ubiquitous ORs in response to conditions associated with concussion might be at the basis for potential abnormal tau phosphorylation possibly through mechanisms associated with the JNK pathway (Fig. VI). This evidence suggests a potential fine-tuning character of OR-mediated JNK regulation in response to conditions associated with concussions.

The sequence of early tau phosphorylation suggests that there are events prior to filament formation that are specific to particular phosphorylated tau epitopes, leading to conformational changes and cytopathological alterations. There is evidence that a healthy neuron develops immunoreactive punctate phospho-tau inclusions, primarily AT8 and AT100, which eventually become preNFTs which give rise to a filamentous intra-cellular inclusion. Eventually the neuron dies and an extra-neuronal NFT, or ghost tangle remains.

Our evidence that the ZINC10915775 may attenuate AT8 and AT100, which are indexes of the early stages of NFT formation, through the activation of OR4M1, supports the hypothesis that certain ligands of ubiquitous OR expression could be developed to target the very early stages of concussion associated tauopathy (Augustinack et al. 2002).

Our observation from the experimental blast-induced concussion model demonstrated, for the first time, a direct cause-and-effect relationship between long-term down regulation of select ORs and a neuropathological feature in concussions. This validates our hypothesis that down regulation of select ORs in PBMCs and in the brains of our human concussion study cohort may be caused by prior concussion exposure (Zhao et al. 2013). Our 3-D *in silico* screening model provides an innovative means to identify novel compounds that can modulate tau phosphorylation through activation of ORs. Future studies will focus on structure-activity relationships (SAR) around the active compound and the optimization of an *in silico* screening system based on this information to identify compounds with higher potency and specificity as potential preventative and possibly therapeutic agents for neuropathological complications in concussions.

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Figure 1. *In silico* 3-D model of OR4M1 and predicted binding pocket

The predicted ligand binding residues from Man, Gilad, & Lancet 2004 are shown in yellow. The predicted binding pocket identified by SiteHound (Ghera & Sanchez 2009) is shown as a red mesh. Additional residues that contribute to the binding pocket, but are not identified in Man, Gilad, & Lancet 2004 are shown in green.

Figure II. Example of virtual screening hit

In panel A, one of the top scoring compounds is shown in cyan as a space-filling model.

Protein binding site side residues are colored as in Figure 1. In panel B, compatibility of the same top scoring compound is shown with predicted binding pocket (red mesh).

Figure III. Structure, docking of the two experimentally validated hits in the OR4M1 binding pocket and dose response assay

In panel A, structure of ZINC10915775; in panel B, predicted binding position for ZINC10915775 compound in the OR4M1 binding pocket; in panel C, dose dependent activation of OR4M1 by ZINC10915775. In panel C, the statistical analysis for the sigmoidal curve was generated by Prism Stat program (GraphPad Software, Inc.); n=3-4 per culture condition.

Figure IV. OR4M1 ligand ZINC10915775 decreased tau phosphorylation and inhibited JNK signaling in primary neurons

Embryonic day 15 cortico-hippocampal neuronal cultures from TgOR4M1 mice (A) and wild type mice (B) were treated with 100 nM ZINC10915775 for 1 hour and western blot analysis were performed to assess tau phosphorylation and JNK signaling. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by two-tailed t-test; $n = 3-4$ per culture condition.

Figure V. Long term effect of blast on the peripheral olfactory receptor levels in a blast-induced rodent model of concussion

Blood was withdrawn at 1, 3, 6, and 12 months following the blast. Quantitative PCR was performed using ABI 7900HT Fast Real-Time PCR System using primers specific for (A) Olr1612; (B) Olr1671; (C) Olr322; (D) Olr735. * $p < 0.05$, $n = 4-5$ rats per group by 2-tailed t-test.

Figure VI.
Scheme of working hypothesis.

Table I

List of primers used in qPCR assays.

Gene	Forward	Reverse
Olr322	AAACTACACCAGGTCAAAGAA	CACCAGGAGAAGGAAGAGAAAC
Olr735	GACCCATCCAGCACATTGAT	GGTTGGTGAGAGGAGAATTGAG
Olr1612	GCCTGTGCCAATACTTTTCTG	CTTGAGTGTTCCTTGAGCATGG
Olr1671	GTGATGTCCTATGACCGCTATG	TGAAGTGCTGAGGTGGTAAAG
TBP	CACCAATGACTCCTATGACCC	CAAGTTTACAGCCAAGATTCACG

Table IIExperimental validation of OR4M1 ligands identified by *in silico* screening

Cluster	ZINC ID	cAMP (% of control) Mean $\pm$ STD
C1	00387281	72.37 $\pm$ 11.49
C2	12916861	81.38 $\pm$ 23.31
	32740605	113.81 $\pm$ 14.83
	09255703	115.59 $\pm$ 25.83
	9578142	88.90 $\pm$ 22.88
	13021362	89.33 $\pm$ 36.94
	09912561	63.98 $\pm$ 19.14
C3	69351464	65.20 $\pm$ 23.20
C4	12974343	71.82 $\pm$ 33.32
	05352464	92.27 $\pm$ 23.98
C5	14148934	107.37 $\pm$ 25.22
	14149349	119.59 $\pm$ 39.55
	10913993	134.95 $\pm$ 28.33
	10913792	102.15 $\pm$ 14.23
C6	03437366	70.56 $\pm$ 13.00
C7	12768603	62.32 $\pm$ 20.52
C8	71896059	47.75 $\pm$ 10.34
C9	05937266	90.38 $\pm$ 3.98
C10	71821329	49.72 $\pm$ 3.08
C11	05455334	62.70 $\pm$ 6.34
C12	65538929	40.86 $\pm$ 5.88
C13	08987000	61.39 $\pm$ 10.79
C14	30481138	82.90 $\pm$ 15.93
C15	10915775	261.89 $\pm$ 39.03 *
C16	32777649	428.61 $\pm$ 46.10 *

two-tailed t-test

*
P< 0.05 vs vehicle control