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"TAARgeting Addiction" The Alamo Bears Witness to Another Revolution:

An Overview of the Plenary Symposium of the 2015 Behavior, Biology and Chemistry Conference

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Abstract

Background—In keeping with the free-thinking tradition San Antonians are known for, the Scientific Program Committee of the Behavior, Biology and Chemistry: Translational Research in Addiction Conference chose trace amine-associated receptor 1 (TAAR1) as the focus of the plenary symposium for its 7th annual meeting held at the University of Texas Health Science Center at San Antonio on March 14 and 15, 2015. The timing of the meeting's plenary session on TAAR1 coincided with the Ides of March, an apt concurrence given the long association of this date with the overthrow of the *status quo*. And whether aware of the coincidence or not, those in attendance witnessed the plunging of the metaphorical dagger into the heart of the dopamine (DA) transporter (DAT)-centric view of psychostimulant action.

Methods—The symposium's four plenary presentations focused on the molecular and cellular biology, genetics, medicinal chemistry and behavioral pharmacology of the TAAR1 system and the experimental use of newly developed selective TAAR1 ligands.

Results—The consensus was that TAAR1 is a DA *and* methamphetamine receptor, interacts with DAT and DA D2 receptors, and is essential in modulating addiction-related effects of psychostimulants.

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Contributors

David Grandy, Gregory Miller and Jun-Xu Li contributed equally to the writing, editing and compiling of the manuscript, and approved the final version.

Conflict of interest statement

All authors declare that they have no conflicts of interest.

Conclusions—Collectively the findings presented during the symposium constitute a significant challenge to the current view that psychostimulants such as methamphetamine and amphetamine solely target DAT to interfere with normal DA signaling and provide a novel conceptual framework from which a more complete understanding of the molecular mechanisms underlying the actions of DA and METH is likely to emerge.

Keywords

Trace amine associated receptor 1; Psychostimulant addiction; Dopamine transporter; Dopamine D2 receptor; Methamphetamine; Schizophrenia; Parkinson's Disease

1. Introduction

One feature that many drugs of abuse have in common is their ability to elevate extracellular dopamine (DA) levels in the brain, an effect that has been correlated with reward-related behaviors (Wise, 1980; Di Chiara and Imperato, 1988). For almost 50 years, researchers interested in trying to understand the physiological and behavioral mechanisms underlying the actions of the psychostimulants such as cocaine, amphetamine (AMPH) and methamphetamine (METH) attempted to identify their primary molecular targets of action. With the publication by Giros et al. (1996) showing that genetically engineered mice lacking (knockout, KO) the DA transporter (DAT) from conception were indifferent to cocaine and AMPH in terms of spontaneous locomotor activity and the release and uptake of DA, the DAT was cast as *the* site of action for both types of stimulants; a view that quickly came to be considered as established fact.

Regrettably, despite numerous challenges, including the increasingly recognized role of vesicular monoamine transporter 2 (Fleckenstein et al., 2007), the predominance of the DAT-centric view of psychostimulant action has hindered attempts to consider, let alone identify and characterize, additional molecular targets of action. The first serious challenges to the DAT-centric dogma of psychostimulant action came in 1998 when Rocha et al. (1998) and Sora et al. (1998) reported that two lines of genetically engineered mice lacking DAT from conception (i.e., a developmental knockout) were still responsive to cocaine. These reports were soon followed by the publication by Carboni et al. (2001) showing that in the DAT KO mice generated by Giros et al. (1996), cocaine and AMPH could still cause significant increases in extracellular DA levels in the nucleus accumbens. That same year Spielewoy et al. (2001), using descendants from the same line of DAT KO mice, reported daily exposure to AMPH resulted in a hypolocomotion phenotype compared to their WT littermates.

In an attempt to resolve the issue, Budygin et al. (2004) and Sotnikova et al. (2004) used descendants from the Giros lineage of DAT KO mice in a series of physiological and behavioral studies. In experiments designed to determine whether or not the reward valence of AMPH differed between WT and DAT KO mice, Budygin et al. (2004) found the absence of DAT did not eliminate AMPH-induced rewarding effect as measured by place conditioning. Furthermore, AMPH retained its ability to increase extracellular DA in the nucleus accumbens of DAT KO mice whereas exposure to the noncatecholic biogenic amine

β-phenylethylamine (PEA), sometimes referred to as the endogenous AMPH (Sabelli et al., 1975; Wolf and Mosnaim, 1983), resulted in the inhibition of novelty-induced locomotion typically displayed by these KO mice, consistent with the idea that sites of action in addition to the DAT, such as a trace amine-sensitive receptor (Boulton et al., 1972; Berry, 2007), could be responsible (Sotnikova et al., 2004).

In 2001, two groups (Borowsky et al., 2001; Bunzow et al., 2001) reported the discovery of a G-protein coupled receptor (GPCR) activated by noncatecholic biogenic amines including the so-called trace amines PEA, *p*-tyramine and DA. In addition the DA metabolite 3-methoxytyramine (3-MT) and the synthetic psychostimulants METH, AMPH and Ecstasy - but interestingly not cocaine - were also reported to be agonists of this new biogenic amine receptor (Bunzow et al., 2001). The significance of the reports by Borowsky et al. (2001) and Bunzow et al. (2001) was acknowledged immediately in commentaries accompanying both articles at the time of their publication (Premont et al., 2001; Kim and von Zastrow, 2001, respectively). Unfortunately, the initial excitement and enthusiasm expressed by many over the discovery of a GPCR directly activated by METH/AMPH but not cocaine was not shared by those individuals tasked with making recommendations to domestic funding agencies, both public and private. As a consequence, in the United States and Canada, the number of laboratories conducting research in the field has remained small.

Fortunately, in Europe this area of research has received sustained and even increasing support over the last decade. In particular, F. Hoffmann-La Roche (Basel, Switzerland) established a drug development program targeting TAAR1 soon after the receptor's discovery. Their program led to the establishment of several non-primate and nonhuman primate animal models for the study of TAAR1-mediated signaling in addition to a collection of receptor-selective compounds including full agonists, partial agonists and an antagonist/inverse agonist as well as polyclonal and monoclonal antibodies against human, mouse, rat and nonhuman primate species of TAAR1 (Bradaia et al., 2009; Revel et al., 2011, 2012a). The availability of these and additional TAAR1-selective reagents (e.g., see www.genecards.org/cgi-bin/carddisp.pl?gene=TAAR1 and www.antibodypedia.com/gene/19712/TAAR1) continues to transform this emerging area of research not only by revealing the consequences of manipulating TAAR1-mediated signaling in cells, tissues and behavior but also by attracting investigators from diverse backgrounds and disciplines to the field.

Perhaps the most consistent *in vitro* observation reported across laboratories conducting TAAR1 research is that the receptor can be directly activated by nanomolar concentrations of DA and METH to stimulate the production of cAMP, in a pertussis toxin-insensitive manner, in heterologous cell-based expression systems. That TAAR1-mediated signaling is directly stimulated by METH/AMPH but not cocaine implies that its activity could contribute to the rewarding/appetitive/interoceptive effects of METH/AMPH that distinguish these drugs from cocaine. As such TAAR1 represents an unconventional yet attractive target against which novel therapeutics designed to treat psychosis, restore the functioning of DA neurons in Parkinson's Disease patients and reduce relapse to the abuse of psychostimulants, and perhaps other drugs such alcohol, nicotine, opiates and cannabis as well, could be developed (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005; Grandy, 2007; Snead et al., 2007; Wolinsky et al., 2007; Berry, 2007; Lindemann et al.,

2008; Ledonne et al., 2010; Revel et al., 2011; Leo et al., 2014; Thorn et al., 2014; Cotter et al., 2015; Miller, 2012).

Since the two first reports 14 years ago (Borowsky et al., 2001; Bunzow et al., 2001), evidence has accumulated that indicates TAAR1-mediated signaling modulates DA levels spatiotemporally in the central nervous system and periphery through a variety of mechanisms involving many different cell types in many anatomical locations (Miller, 2011). It was the intent of this symposium to stimulate interest in and discussion about this emerging area by bringing together leaders in the field to share their latest research findings in the context of psychostimulant abuse with a diverse audience composed of students, postdoctoral fellows, clinicians and basic scientists. It was in this setting that the role of TAAR1-mediated signaling as a function of psychostimulant (cocaine and AMPH/METH) exposure was explored. [Footnote: Four speakers presented at the symposium. Besides the three authors of this review, Dr. Giuseppe Cecere, a medicinal chemist from F. Hoffmann-La Roche, also gave a talk regarding the medicinal discovery and development of selective TAAR1 ligands. However, he did not contribute to the preparation of this review due to time conflict.]

2. TAAR1-DAT INTERACTIONS: *IN VITRO AND EX VIVO* EVIDENCE

TAAR1 has a wide agonist spectrum that includes endogenous amines - both the common biogenic amines (DA; norepinephrine, NE and serotonin, 5HT) and trace amines (e.g., PEA, tyramine, octopamine, synephrine and tryptamine) as well as structurally-related AMPH-like compounds (AMPH, METH and MDMA; Bunzow et al., 2001; ractopamine, Liu et al., 2014), and thyronamines (Scanlan et al., 2004; Hart et al., 2006; Wainscott et al., 2007; Xie et al., 2007b). The receptor's wide-spread expression in many different types of neurons (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann et al., 2008; Espinoza et al., 2015), astrocytes (Cisneros and Ghorpade, 2014), immune cells (D'Andrea et al., 2003; Nelson et al., 2007; Wasik et al., 2012; Panas et al., 2012; Sriram et al., 2015) and cells of the cardiovascular system (Scanlan et al., 2004; Frascarelli et al., 2008; Broadley et al., 2009) suggests TAAR1-mediated signaling subserves one or more fundamental roles in cellular physiology (Grandy, 2007; Xie et al., 2007b; Lindemann et al., 2008).

With the reports of its discovery and deorphanization in 2001 (Borowsky et al., 2001; Bunzow et al., 2001), researchers were challenged with expressing and characterizing the receptor *in vitro* as it is mostly sequestered intracellularly (Bunzow et al., 2001, Miller et al., 2005; reviewed in Grandy, 2007 and Miller, 2011). Functional assays, immunocytochemical detections and biotinylation experiments all suggest that the majority of the receptor protein remains associated with membranes in the intracellular milieu (Borowsky et al., 2001; Xie et al., 2007, 2008; Szumska et al., 2015). The biological significance of this cellular distribution remains enigmatic.

When the rhesus monkey TAAR1 was cloned (Miller et al., 2005), questions still remained as to the receptor's role in DA neurons. The intracellular sequestration of TAAR1 in transfected cells and the absence of availability of specific pharmacological probes presented challenges for pharmacologically characterizing TAAR1. Since DA, PEA,

tyramine, METH and AMPH are DAT substrates one interesting hypothesis born out of these challenges is that the DAT could serve as a conduit by which TAAR1 agonists gain access to an intracellular receptor. To test this idea *in vitro* and *ex vivo* radioligand uptake and CRE-luciferase receptor assays were developed (Miller et al., 2005). Once these assays were validated functional interactions between DAT and TAAR1-mediated signaling could be directly evaluated. Indeed, a dramatic potentiation of TAAR1 signaling through the cAMP/PKA pathway in response to DA, noncatecholic biogenic amines, METH and AMPH, is robust and consistently shown in *in vitro* assays using heterologously-expressed DAT, TAAR1 and CRE-Luc reporter in HEK293 cells (Miller et al., 2005; Xie et al., 2007b; Xie and Miller, 2008). A similar phenomenon is seen in both NE transporter (NET) and 5-HT transporter (SERT)-transfected TAAR1-expressing HEK-293 cells as well. Furthermore, this response could be recapitulated in striatal (DAT and SERT) and thalamic (NET) synaptosomes (Xie et al., 2008b). Enhanced TAAR1 signaling in response to DA, noncatecholic biogenic amines and METH in the presence of a monoamine transporter was found to be blocked by selective as well as nonselective monoamine transporter inhibitors.

Following the demonstration that the presence of DAT enhances TAAR1-mediated signaling the effects of TAAR1 signaling on DAT kinetic functions (i.e., uptake and efflux) were investigated (Xie and Miller, 2007; Xie et al., 2008b; Xie and Miller, 2009). *In vitro* the responses observed using HEK293 cells co-expressing DAT and TAAR1 are dependent on ligand concentration, in a manner consistent with the idea that TAAR1 is mostly sequestered intracellularly (Xie and Miller, 2007). When TAAR1 was absent, DAT uptake of 10 nM [^{3}H]DA was competitively inhibited by the presence of cold substrate. In contrast, in the presence of TAAR1 a time-dependent inhibition of uptake beyond what is observed in DAT-only cells occurs. Only this additional inhibition is blocked in the presence of either the protein kinase A (PKA) inhibitor H89 (10 μM) or the protein kinase C (PKC) inhibitor Ro32–0432 (10 μM), returning the level of inhibition back to that matching the DAT-only cells (Xie and Miller, 2007).

Efflux studies have also been performed wherein co-transfected cells expressing either DAT with TAAR1 or DAT alone were loaded with [^{3}H]DA and then exposed to cold substrate. At a very low preloading concentration of 10 nM [^{3}H]DA (a concentration of DA insufficient to activate TAAR1 (Borowsky et al., 2001; Bunzow et al., 2001; Xie and Miller, 2007), excess substrate/agonist significantly promoted efflux but only in DAT/TAAR1 cells, whereas at a higher preloading concentration of 1 μM [^{3}H]DA, substrates cause efflux of [^{3}H]DA from DAT-only cells, as reported by other laboratories (reviewed in Miller, 2011) and a greater efflux occurs in cells co-expressing DAT/TAAR1 compared to those expressing DAT alone. Similar to the uptake experiments, this additional efflux can be inhibited in the presence of a PKC inhibitor, returning the level of efflux back to that displayed by the DAT-only cells.

The interpretation made of these data is that as a TAAR1 ligand/DAT substrate (i.e. METH/AMPH/DA) enters the cell via the DAT and accumulates, TAAR1 signaling progressively occurs. In turn, phosphorylation cascades are promoted ultimately modulating DAT kinetics. These studies gave the first indication that TAAR1 could serve to trigger phosphorylation events that are well-recognized as important modulators of DAT function. In this regard,

PKC-mediated internalization of DAT is a well-recognized mechanism of DAT regulation (Pristupa et al., 1998). A role for TAAR1 in triggering DAT internalization was demonstrated in DAT biotinylation assays using the same cell and synaptosome preparations (Xie and Miller, 2009). METH reduced cell surface DAT levels in TAAR1-DAT cells (but not in DAT cells), and also reduced surface DAT levels in wild-type mouse striatal synaptosomes and rhesus monkey synaptosomes, whereas only a small (yet significant) decrease in surface DAT was observed in TAAR1 knockout mouse striatal synaptosomes. Furthermore, TAAR1-mediated DAT internalization was blocked by the addition of the selective cell-permeable PKC inhibitor, Ro32–0432, further supporting the hypothesis that TAAR1 signaling can function as a triggering mechanism for the PKC-driven cellular events which hallmark DAT internalization.

While other GPCRs can signal through cAMP/PKA and/or Ca^{++}/PKC/NFAT pathways in DA neurons similar to TAAR1 (Panas et al., 2012), TAAR1 possesses unique attributes that make it relevant to DA neuron function and consequently, a target of novel therapeutic drugs that can regulate DA. Since TAAR1 is activated by DA (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005; Xie et al., 2007b) and it is expressed in DA-producing neurons, one corollary is that it could serve as a DA autoreceptor. However, if it were to serve such a function TAAR1 would most likely do so through a different mechanism than is regulated by the DA D2 autoreceptor (D2R) because TAAR1 is thought to be $G\alpha_s$-linked.

Indeed, TAAR1 signaling has been shown to functionally oppose DA D2R signaling by activating the cAMP/PKA pathway (Xie et al., 2007). In this regard, it was speculated that the balance of TAAR1 and DA D2R–mediated signaling is an important regulatory mechanism in DA neurons. This original observation of TAAR1 and DA D2R interaction has subsequently been confirmed and expanded upon with observations that both receptors can heterodimerize with each other under certain conditions (Espinoza et al., 2011; Salahpour et al., 2012; Espinoza et al., 2015; Harmeier et al., 2015). Furthermore, TAAR1 has much higher affinity for trace amines than the DA D2R (Wainscott et al., 2007; Xie and Miller, 2008). This has implications for how noncatecholic biogenic trace amines might influence the balance between TAAR1 and DA D2R signaling by DA.

The fact that TAAR1 is a direct, high affinity target for METH and AMPH whereas the DA D2R is not (Xie et al., 2007b) supports the idea that METH/AMPH interferes with TAAR1/DA D2R functional and/or physical interaction(s). Additional DA D2R/TAAR1 interactions with functional consequences are revealed by the results of experiments demonstrating that in addition to the cAMP/PKA pathway (Panas et al., 2012) stimulation of TAAR1-mediated signaling is linked to activation of the Ca^{++}/PKC/NFAT pathway (Panas et al., 2012) and the DA D2R–coupled, G protein-independent AKT/GSK3 signaling pathway (Espinoza et al., 2015; Harmeier et al., 2015), such that concurrent TAAR1 and DA DR2R activation could result in diminished signaling in one pathway (e.g. cAMP/PKA) but retention of signaling through another (e.g., Ca^{++}/PKC/NFA), as *in vitro* and *ex vivo* experiments had originally suggested (Xie et al., 2008b; Panas et al., 2012).

The characterization of cloned mouse, rat, rhesus and human species of TAAR1 heterologously expressed in cultured, immortalized cells has in the past and will in the future

continue to significantly contribute to what is known about TAAR1's pharmacology and physiology. However, to understand the receptor's role in behavior one must turn to whole animal models. Unfortunately, for those interested in TAAR1 this presented a challenging prospect because at the time of its discovery there were no known selective reagents available or animal models to draw on.

Several mouse lines and one rat line have so far been genetically engineered to be deficient in TAAR1 (Seeman et al., 2006; Wolinsky et al., 2007; Lindemann et al., 2008; Revel et al., 2011; Di Cara et al., 2011; see also the Knock Out Mouse Project, UC Davis, CA; www.komp.org), overexpress TAAR1 (Revel et al., 2012b) or express a reporter driven by the TAAR1 gene promoter (Lindemann et al., 2008)

At the molecular level the most consistent findings are that TAAR1 is expressed in DA neurons of the ventral tegmental area (VTA; Borowsky et al., 2001; Bunzow et al., 2001; Lindemann et al., 2008; Baradia et al., 2009; Revel et al., multiple publications) and that in the complete absence of TAAR1 a significant alteration in the physiology of central DA neurons occurs. For example, the percentage of high-affinity DA D2Rs ($D2^{High}$) in the striata of *taar1* KOs is greater than their WT littermates (Seeman et al., 2006; Wolinsky et al., 2007) as is DA D2R transcription, translation and signaling (Espinoza et al., 2015). The absence of TAAR1-mediated signaling also results in VTA DA neuron hyperactivity (Lindemann et al., 2008; Bradaia et al., 2009; Revel et al., 2011; 2012a; 2012b); decreased DAT functionality (Xie and Miler, 2009); changes in DA-related brain neurochemistry (Leo et al., 2014); a preference for ethanol (Lynch et al., 2013); and behavioral supersensitivity to the psychostimulants AMPH, METH (Wolinsky et al., 2007; Lindemann et al., 2008; Achat-Mendes et al., 2012) and cocaine (Revel et al., 2011; Jing and Li, 2014).

In the context of drug reinforcement, conditioned place preference (CPP) studies have revealed mice deficient in TAAR1 acquired methamphetamine-induced CPP earlier than WT mice and retained CPP longer during extinction training while no differences were observed in morphine-induced CPP (Achat-Mendes et al., 2012). This differential effect may involve DA in that DA released by AMPH or METH interacts with DA D1 (and DA D2) receptors to establish CPP (Hiroi et al., 1991), whereas DA D1 receptors are reportedly not required for morphine-induced CPP (Urs et al., 2011). Similar to the augmented responses to AMPH and METH, mice lacking TAAR1 also showed significantly greater preference for and consumption of ethanol in a two-bottle choice (TBC) paradigm, with no significant difference observed in TBC with sucrose (Lynch et al., 2013). As is the case for AMPH and METH, DA is implicated in ethanol-related behaviors (e.g., El-Ghundi et al., 1998; Heidbreder et al., 2007). Together, the pharmacogenic phenotypes observed in TAAR1-deficient mice and the ability of TAAR1 activation to regulate DA suggest that TAAR1 is a modulator of DA-mediated rewarding effects of drugs of abuse. Notably, this includes AMPH-like psychostimulants such as METH, which directly binds to the receptor, as well as ethanol (and cocaine, as discussed below), which may indirectly alter TAAR1 signaling via the drug's ability to affect levels of endogenous DA. While these findings have been consistent and fitting with the hypothesis that TAAR1 may exert inhibitory actions on the physiological and behavioral effects of drugs of abuse via its ability to regulate DA, studies in TAAR1-deficient animals cannot fully inform on the receptor's biological role(s)

in the intact animal. Consequently, with the publication of the first selective TAAR1 antagonist, EPPTB, by Bradaia et al. (2009) and the development of specific TAAR1 agonists, the field was transformed.

3. THE DISCOVERY OF THE FIRST SELECTIVE TAAR1 ANTAGONIST AND ITS SEQUELAE

The discovery of EPPTB came out of Hoffmann-La Roche's program to develop selective TAAR1 compounds as novel research tools and therapeutics for understanding and treating psychiatric (e.g. schizophrenia) and neurodegenerative (e.g., Parkinson's Disease) disorders. The Roche compound library (~788,000 compounds) was screened using a high throughput functional (cAMP production) assay wherein a human-rat TAAR1 chimera (Reese et al., 2007) was expressed in HEK-293 cells (Stalder et al., 2011). Initially all compounds were evaluated for their agonist and antagonist activity. Those with the best activity profiles constituted a primary hit set of structurally similar molecules. Based on these results 25 compounds were further evaluated for their agonist and antagonist properties at mouse TAAR1 (mTAAR1) heterologously expressed in HEK-293 cell. Of the different chemical classes represented in the expanded hit set the benzanilides displayed the lowest IC_{50}'s (< 1 μM) and the highest EC_{50}'s (>20 μM) as a class, behaving as antagonists of 1.5 μM PEA-activated TAAR1-mediated cAMP production (Bradaia et al., 2009). Of the 12 benzanilides with IC_{50}'s < 1 μM two were selected for further analysis in a proprietary binding assay that exposed membranes prepared from HEK-293 cells expressing mouse, rat or human TAAR1 to the radioligand [^{3}H]-rac-2-(1,2,3,4-tetrahydro-1-naphthyl)-2-imidazoline with specific binding defined in the presence of 10 μM cold compound (Bradaia et al., 2009).

This analysis revealed that the binding profiles of the two lead compounds were completely dependent on the species of TAAR1 under investigation. As a consequence the goal of future lead optimization efforts shifted from developing a novel therapeutic to producing a useful research tool. Since the most active members of the series were poorly soluble in water and rapidly degraded, subsequent medicinal chemistry efforts focused on improving these attributes and led to (N-(3-ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB; RO5212773; Bradaia et al., 2009). EPPTB has a high affinity for mTAAR1 (9 nM), low IC_{50} (28 nM) with respect to PEA-stimulated cAMP production and reduced clearance while still able to attain a respectable brain/plasma ratio in mice when administered intraperitoneally (Stalder et al., 2011).

Prior to EPPTB's discovery two lines of genetically engineered *taar1*-deficient mice (KOs) had been generated and characterized (Wolinsky et al., 2007; Lindemann et al., 2008). According to both groups wild type (WT) and KO animals are surprisingly similar in most aspects with the exception of an unexpected hypersensitivity of *taar1*$^{-/-}$ mice to the locomotor-stimulating effects of AMPH despite their 'normal' levels of accumbal catecholamines. Of these two reports, Lindemann et al. took a more mechanistic approach reporting the findings of their electrophysiological experiments demonstrating VTA DA neurons completely lacking TAAR1 display a significant decrease in the inter-event interval time manifesting as a significant increase in spontaneous firing rate (Lindemann et al., 2008).

Not surprisingly then, the first publications documenting EPPTB's effects on TAAR1-mediated signaling report the results of *in vitro* electrophysiological experiments using slice preparation containing VTA DA neurons from wild type (WT) and *taar1* KO mice (Bradaia et al., 2009; Revel et al., 2011). While the antagonist had no effect in DA neuron-containing midbrain slices prepared from *taar1* KO mice, exposing corresponding slices prepared from WT mice to EPPTB resulted in an electrophysiological phenotype indistinguishable from *taar1* KO mice. They then went on to show that TAAR1-mediated signaling is constitutive, exerting a persistent inhibitory tone by hyperpolarizing the plasma membranes through the modulation of intracellular potassium ion concentration; not unlike the effect produced by activation of DA D2R auto-receptors (Bradiaia et al., 2009; Espinoza et al., 2011, 2015).

That TAAR1 signaling is coupled to the inhibition of VTA DA neuron firing was a surprising finding. Being a receptor coupled to $G_{\alpha}s$ its activation was expected to contribute to membrane depolarization. Also, earlier data suggested that TAAR1 activation leads to inhibition of DA uptake by DAT, promotion of DA efflux by DAT and DAT internalization which presumably would augment extracellular DA levels, whereas inhibition of DA neuronal firing rate would likely decrease extracellular DA levels, albeit that DAT localization is reportedly uniformly distributed in the plasma membrane of the soma, the neuronal extensions, and varicosities along these extensions in DA neurons (Eriksen et al., 2009) which may differ from sites of DA release in response to neuronal firing. Nonetheless, this mismatch in expectations immediately attracted the attention of those trying to determine whether TAAR1 is a METH/AMPH and DA receptor *in vivo* as it is *in vitro* (Bunzow et al., 2001; Wainscott et al., 2007; Xie and Miller, 2009; Panas et al., 2012). Consequently, it was considered fundamental to employ EPPTB in a traditional behavioral pharmacology approach designed to identify DA and METH-activated TAAR1-coupled actions in the whole animal.

4. FROM *IN VITRO* PHENOMENOLOGY TO BEHAVIORAL PHARMACOLOGY: THE INTEGRATION OF TAAR 1-MEDIATED SIGNALING AND DRUG ADDICTION

Psychostimulants such as METH produce several well-documented behavioral effects in humans, nonhuman primates and rodents including locomotor hyperactivity, intravenous (IV) drug self-administration, conditioned place preference and sensitization (Vezina, 2007); all of which have a significant DA component (Wise, 1980). Therefore, if it could be demonstrated that METH-activated TAAR1-mediated signaling contributes to any of these behavioral outcomes it would constitute a significant challenge to the notion that DAT is the only important target of METH/AMPH action.

In one effort to test this hypothesis EPPTB's effect on the spontaneous locomotor activity in a familiar environment displayed by WT and *taar1*-deficient mice chronically exposed to 3 mg/kg METH (i.p.) over a range of doses was examined. The results of this study (Grandy, unpublished observations; SfN abstracts) support the interpretation that EPPTB prevents to a significant degree METH-stimulated locomotor activity but only in WT mice with a history of chronic METH exposure. The ability of EPPTB to reduce METH-stimulated locomotor

activity in mice chronically exposed to METH is the opposite of what was expected given previously published reports of locomotor activity experiments involving the manipulation of TAAR1-mediated signaling in rodents (Revel et al., 2011, 2012a, 2012b) acutely exposed to METH. Rather than discounting their findings as an anomaly Grandy advocated the view that this discrepancy in expectations demands a mechanistic explanation. In the model he proposed, acute and chronic exposure to METH differentially affect the receptor's cellular distribution and as a consequence, its signaling. One of the corollaries of his model is that a partial agonist will diminish METH-stimulated behaviors such as locomotor sensitization and relapse to intravenous self-administration. In support of his prediction both of these 'sensitized' behaviors were recently reported to be attenuated by the TAAR1 partial agonist RO5263397 (Jing et al., 2014 and below). Clearly a better understanding of the TAAR1-sensitive molecular mechanism(s) underlying the behavioral manifestations of METH's actions will benefit from additional studies.

Although EPPTB's capacity to significantly diminish METH-stimulated locomotor activity represents a significant challenge to the DAT-centric dogma of psychostimulant action, this is not an animal model of drug addiction *per se*. Animal models that are better validated such as IV METH self-administration by rodents and nonhuman primates, a model that is considered the gold standard by those working in this field, should be used to confirm the effects of EPPTB. Despite its high affinity for mouse TAAR1 and desirable plasma/brain distribution profile EPPTB is technically challenging to work with *in vivo* preventing many, who otherwise would have, from doing so (Grandy, unpublished observations).

Fortunately, this *status quo* ended when Revel et al. (2013) published the structure/activity profile of RO5263397 - an orally available, high affinity, selective, partial TAAR1 agonist that is soluble in standard research vehicles. RO5263397 displays low nanomolar potency in functional assays, and possesses no adverse pharmacokinetic properties while it is capable of penetrating the blood brain barrier. Although the synthetic route to RO5263397 is straightforward it results in a racemic mixture of two enantiomers. The two isomers can be chromatographically separated but for behavioral experiments this is probably not necessary since only the 'S' form has biological activity, attenuating cocaine-stimulated locomotor activity in rodents (Revel et al., 2013).

Encouraged by the report by Revel et al. (2013), Thorn et al. (2014a, 2014b) administered RO5263397 to rats and found that it significantly blocked the induction and expression of locomotor sensitization to cocaine. Similar attenuation was also found using different Roche TAAR1-selective agonists (RO5263397 and RO5203648) in the expression of behavioral sensitization to METH (Jing et al., 2014; Cotter et al., 2015). These results demonstrate that TAAR1-mediated signaling contributes significantly to cocaine- and METH-induced behavioral plasticity and related long-lasting behavioral maladaptations. The relevance of TAAR1 in the mediation of drug reward and reinforcement was further examined using conditioned place preference (CPP) and IV drug self-administration paradigms. In these settings RO5263397 significantly attenuates the expression of cocaine CPP (Thorn et al., 2014a). This finding is in agreement with a prior report by Revel *et al*. who reported the TAAR1 partial agonist RO5203648 could reduce cocaine self-administration in a simple fixed ratio (FR)1 schedule of reinforcement (Revel et al., 2012b). In addition, more recently

Pei et al. (2015) used the intracranial self-stimulation (ICSS) paradigm and found that the TAAR1 full agonist RO5256390 and partial agonist RO5263397 dose-dependently prevented cocaine-induced lowering of ICSS thresholds, suggesting a *bona fide* reduction of cocaine-induced reinforcing effects.

Although the FR1 schedule of reinforcement is most often used in IV self-administration paradigm to demonstrate the motivational aspect of drug consumption, in the behavioral economic approach the FR is systematically increased until the subject fails to earn a reinforcer thereby providing a metric of how hard the individual is willing to work for the reinforcer (i.e. reinforcing effectiveness of the drug). In behavioral economic analysis the increased elasticity of a demand curve is an indication that the commodity (e.g., an injection of cocaine) is less reinforcing so consumption decreases more quickly when the price (FR) increases (Hursh et al., 2005; Thorn et al., 2014a).

In behavioral economic experiments involving rats where the TAAR1 partial agonist RO5263397 was used the elasticity of the cocaine demand curve increased, consistent with the interpretation that RO5263397 is effective in attenuating cocaine's reinforcing effects (Thorn et al., 2014). In a similar progressive ratio (PR) schedule of reinforcement the TAAR1 partial agonist RO5203648 decreased cocaine-maintained responding in a dose- and time-dependent manner while delaying the time to breakpoint for cocaine iv self-administration (Pei et al., 2014). RO5203648 has also been reported to attenuate METH IV self-administration under a FR 1 schedule and did not sustain self-administration in rats previously trained to self-administer METH IV (Cotter et al., 2015). Similarly, RO5263397 could significantly reduce METH IV self-administration in rats (Jing et al., 2014).

It is worth noting that the TAAR1 partial agonists studied thus far (i.e. RO5263397 and RO5203648) increase the firing rate of midbrain DA neurons in *ex vivo* slices prepared from drug naïve WT mice (Revel et al., 2012b, 2013) but in rodents with a history of chronic exposure to psychostimulants they reduce METH IV self-administration (Thorn et al., 2014a; Jing et al., 2014; Cotter et al., 2015). Unexpected and discordant results often foretell the revelation of new insights, if only they were investigated further. Accordingly more work is needed to fully understand this discrepancy.

Together, the above findings indicate that TAAR1 plays a fundamental role in modulating the rewarding and reinforcing properties of cocaine and METH by their indirect and direct activation of TAAR1-mediated signaling that in some way manifests as a reduction in drug intake, an important therapeutic goal to control drug consumption.

A major obstacle to successfully overcoming drug addiction is the high relapse rate even after prolonged abstinence. Relapse to compulsive drug use can be triggered by diverse events including re-exposure to drugs in environmental and/or social contexts previously associated with drug-taking or stress (Shaham et al., 2003). Pei et al (2014) used a context-induced relapse model to examine the impact of TAAR1 agonism on relapse to cocaine-seeking behavior. Both the TAAR1-selecitve partial agonist RO5203648 and full agonist RO5256390 dose-dependently suppressed cocaine seeking after a 2-week period of forced abstinence from chronic cocaine self-administration. Consistent results were also obtained in

an extinction-reinstatement model using RO5203648 (Pei et al., 2014). In a similar paradigm the TAAR1 partial agonist RO5263397 attenuated both drug-associated cue- and cocaine prime-induced reinstatement of drug-seeking behavior (Thorn et al., 2014a). Importantly, a similar prevention of cue-induced and drug-primed relapse to METH IV self-administration was observed with RO5263397 without altering cue-induced reinstatement of sucrose-seeking behavior (Jing et al., 2014). These data provide strong evidence in support of the hypothesis that pharmacological agonists of TAAR1-mediated signaling has the potential to become an effective therapeutic adjunct to comprehensive approaches to prolonging abstinence from psychostimulant abuse.

5. CONCLUSIONS

This was a historic conference as it was the first meeting devoted primarily to TAAR1. It also provided the first opportunity to seriously discuss the concept that TAAR1 is a high-affinity receptor for METH/AMPH *and* DA in the context of a new molecular model of psychostimulant action.

With the focus being on the receptor's role in drug addiction the four speakers presented data that demonstrated to the satisfaction of the audience TAAR1-mediated signaling is activated *in vivo* by the synthetic psychostimulants METH and AMPH *as well as* DA, the endogenous trace amines and thyronamines. Furthermore, they showed that selective modulation of TAAR1-mediated signaling using small molecules affects DA D2R–mediated signaling as well as DAT functions with significant consequences in the context of drug-taking and drug-seeking behaviors. When considered together with the rapidly growing literature in the field a compelling case emerges in support of developing TAAR1-selective agonists as medications for preventing relapse to psychostimulant abuse.

In the course of pursuing new opportunities there are always obstacles to be overcome that often can only be surmounted by a revolution. For English speaking settlers in the Mexican Province of Texas cultural and political differences with the Centralist government of the Republic of Mexico eventually resulted in such a revolt. The turning point in what came to be called the Texas Revolution (1835–1836) was when President General Antonio López de Santa Anna's Mexican army defeated and brutally killed all the "Texians" defending the small garrison at Alamo Mission (March 6, 1836). Outrage over Santa Anna's slaughter of the brave Texians who stood defiant against the Mexican invaders ignited an urge for revenge and rallied by the cry "Remember the Alamo!" the Texian army, led by Sam Houston, soon thereafter defeated and captured Santa Anna.

One lesson from this history is that the sacrifice of a few can make a significant difference for many. The same sentiment is apropos to TAAR1 - a handful of scientists commit *all* of their resources and credibility to achieve a better understanding of TAAR1 biology in health and disease but their research programs were killed for lack of support.

However, thanks to the scientific organizers, those who attended this meeting were given the opportunity to learn that TAAR1 is a critical component of the greater DA system and as such has to be included in any new model attempting to interpret experimental data related

to the neurochemistry of acute and chronic psychostimulant action. A continued reluctance by public and private funding agencies to support future research in this area is tantamount to a blatant disregard for a promising therapeutic approach as well as the needs of those struggling with psychostimulant dependence, their families and their communities.

Consequently, when thinking about DA, “Remember TAAR1!”

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Highlights

- Recent progress on trace amine-associated receptor 1 (TAAR 1) was described
- Historical perspective of TAAR 1 and its interaction with dopaminergic system was reviewed.
- Both *in vitro* and *in vivo* evidence support the critical role of TAAR 1 in drug addiction. #body