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The Emerging Role of Trace Amine Associated Receptor 1 in the Functional Regulation of Monoamine Transporters and Dopaminergic Activity

Gregory M. Miller
The New England Primate Research Center, Harvard Medical School, Southborough, MA 01772, 508-624-8023

Gregory M. Miller: gmiller@hms.harvard.edu

Abstract

It is now recognized that Trace Amine Associated Receptor 1 (TAAR1) plays a functional role in the regulation of brain monoamines and the mediation of action of amphetamine-like psychostimulants. Accordingly, research on TAAR1 opens the door to a new avenue of approach for medications development to treat drug addiction as well as the spectrum of neuropsychiatric disorders hallmarked by aberrant regulation of brain monoamines. This overview focuses on recent studies which reveal a role for TAAR1 in the functional regulation of monoamine transporters and the neuronal regulatory mechanisms that modulate dopaminergic activity.

The Trace amines have been investigated in mammalian brain for about 40 years (Boulton, 1971) and their existence has been known of since *p*-tyramine was first identified and synthesized in 1865 (Schmitt and Nasse). Boulton (1971) first referred to these biogenic amines as “microamines”, a term meant to imply that their presence, particularly in brain, was very low compared with the common biogenic amines. The term “trace amine”, adopted in 1975 during a study group at the American College of Neuropharmacology meeting that year, is somewhat of a misnomer because certain trace amines are present in microgram amounts in some species and in some tissues and following treatment with monoamine oxidase inhibitors, whereas the classic common biogenic amines are present in certain tissues and brain regions in trace amounts (Boulton, 1984).

The trace amines are metabolized by monoamine oxidase (MAO) and have high turnover rates. β-PEA is highly selective for MAO-B whereas tyramine and other trace amines are metabolized by both MAO-A and MAO-B (Philips and Boulton, 1979; Durden and Philips 1980). β-PEA and tyramine were shown to possess turnover rates that were so fast that in unit time, quantities similar to those existing for dopamine, norepinephrine and serotonin were thought to be synthesized (Wu and Boulton, 1973; 1974; 1975; Durden and Philips 1980). The trace amines were known to exist in brain at very low levels(<10 nM), but particularly noted decades ago was that trace amine levels were elevated in depression and other neuropsychiatric disorders (Coppen et al, 1965; Boulton, 1976; Sandler et al, 1979; O’Reilly et al 1991; reviewed in Berry, 2004 and Branchek and Blackburn, 2003). Trace amines, and in particular β-phenylethylamine (β-PEA), were also known to be implicated in brain reward circuitry and the reinforcing properties of psychostimulants (Biel, 1970; Sandler and Reynolds, 1976; Stein, 1964; Risner and Jones, 1977; Shannon and Thompson, 1984; Gilbert and Cooper, 1983). β-PEA and other trace amines were recognized as substrates for monoamine transporters, suggesting that extracellular levels of trace amines may be regulated in tandem with biogenic amines (Segonzac et al, 1985; Meiergerd and Schenk, 1994; Burnette et al, 1996; Sitte et al, 1998; Li et al, 2002; Miller et al., 2005). However, trace amines may not be primarily stored in vesicles. β-PEA, tyramine and

tryptamine were not released in response to K^+-induced depolarization (Dyck, 1988; Dyck, 1989; Henry et al, 1988). Tissue levels of β-PEA were not depleted and tissue levels of tyramine were only moderately decreased following displacement of synaptic vesicle stores with reserpine (Boulton et al, 1977; Juorio et al, 1988).β-PEA and tryptamine are more lipophilic than tyramine, and their release and regulation were proposed to be a function of their ability to cross cell membranes (Mack and Bonisch, 1979). Also, the trace amines elicited electrophysiological responses distinct from those of classical biogenic amines (Boulton and Dyck, 1974; Juorio, 1976; Henwood et al, 1979; Jones et al, 1980; Durden and Philips, 1980; Parker and Cubeddu, 1988; Grimsby et al, 1997). Reports of specific binding sites for [^{3}H]β-PEA, [^{3}H]tryptamine and [^{3}H]tyramine in mammalian brain that display a unique pharmacology and localization had also appeared (Ungar et al, 1977; Hauger et al, 1982; Kellar and Cascio, 1982; Perry, 1986; Altar et al, 1986; Vaccari, 1986), although the binding site for β-PEA was subsequently shown to most likely represent binding to MAO (Li et al., 1992). Although binding site density correlated with levels of endogenous trace amines (Karoum et al, 1981; Hauger et al, 1982), the functional relevance of these trace amine binding sites remained elusive, and without selective pharmacological agents or cloned receptors, interest in trace amine binding sites had gradually waned throughout the 1980's and 1990's.

In the absence of unique targets for these putative neuromodulators, the contribution of trace amine function to the rewarding properties of abused drugs or to neuropsychiatric disorders was questionable, and progress and wider interest in the field slowed. It was not until the first reports of a bona fide mammalian trace amine receptor by Borowsky et al (2001) and Bunzow et al (2001) appeared that a revitalization of an old interest in brain trace amines as neuromodulators occurred. It suddenly became feasible to untangle the potentially unique contributions of trace amines and their receptors to brain function and amphetamine-like psychostimulant responsivity.

Cloning, expression and pharmacological assessment of TAAR1

In 2001, a family of intronless G protein-linked trace amine associated receptors (TAARs)was cloned from human and rodent tissues (Borowsky et al., 2001). Fifteen G protein-coupled receptors (GPCRs) in all were identified. Although the degree of homology between receptors within a species was extremely high, the degree of amino acid identity among orthologues was relatively low, suggesting that although these receptors are relatively recent expansions of the genome, they are evolving at a rapid rate(Lindemann et al., 2005; Vallender et al., 2010). There was also a larger number of rat receptors identified as compared with human receptors (four human receptors versus 14 rat receptors).Among all receptors cloned from human and rodent, only two, TA_1 and TA_2 (here referred to as TAAR1 and TAAR4, respectively, based on a nomenclature proposed by Lindemann et al., 2005) were found to bind and/or be activated by biogenic amines. In voltage clamp experiments, octopamine, and more weakly dopamine and serotonin, elicited inward currents at 100 μM in oocytes expressing rat TAAR1 along with the cAMP-responsive Cl channel, CFTR. Similar currents were generated by tyramine at a lower concentration (100 nM). These initial results suggested that stimulation of rat TAAR1 by trace amines resulted in the generation of cAMP leading to CFTR channel opening, presumably via activation of the endogenous Xenopus G protein $G_{\alpha s}$. Further studies in human TAAR1-transfected COS-7 cells demonstrated that human TAAR1 was activated most potently by β-PEA and tyramine, and more weakly by octopamine and dopamine, as indicated by cAMP content measurements following a 10 min incubation. Radioreceptor assays with [^{3}H]tyramine demonstrated high-affinity, saturable binding in human TAAR1-expressing cell membranes, allowing determination of the rank order of potency for various monoamines. The rank order of potency was similar between the binding assays and functional assays, although the K_i

values determined from binding displacement were about 6-fold lower than the EC_{50} values determined in the functional studies (β-PEA and tyramine > dopamine and octopamine > tryptamine, histamine, serotonin and norepinephrine). The expression of rat TAAR4 in COS-7 cells resulted in a more modest increase in cAMP accumulation as well, but only in response to β-PEA and tryptamine. There were no functional responses to tyramine, β-PEA, tryptamine, octopamine or the classical biogenic amines observed in COS-7 cells expressing other TAAR family members(Borowsky et al., 2001).

Directly following the report of Borowsky et al (2001), Bunzow et al (2001) reported the cloning of rat TAR1 (here referred to as TAAR1). These investigators demonstrated that rat TAAR1 was activated by a wide spectrum of agonists. *p*-Tyramine and β-PEA elicted cAMP production as did other trace amines, dopamine and dopamine analogs, a variety of drugs that are known to bind to dopamine, adrenergic and serotonergic targets, and in particular, ergolines and amphetamine-like drugs of abuse (Bunzow et al., 2001). With regard to molecular targets for drugs of abuse, the discovery that rat TAAR1 was activated not only trace amines but also by drugs of abuse such as amphetamine and MDMA, was a remarkable finding, in that it demonstrated a novel direct molecular target for these drugs in brain that was a GPCR rather than a monoamine transporter.

The pharmacological evidence of rat TAAR1 as a direct target for drugs of abuse coupled with the high divergence in gene sequence between the rodent and human receptors led Miller et al (2005) to investigate TAAR1 in rhesus monkeys, a species of close evolutionary proximity to humans. TAAR1 cloned from rhesus monkey shared a 96% sequence homology to human TAAR1 (versus ~79% with rodent). As was the case with rat TAAR1, trace amines and amphetamine-related drugs stimulated cAMP accumulation in rhesus monkey TAAR1-transfected cell lines, as measured by a CRE-luciferase reporter assay (Miller et al., 2005; Xie et al., 2007).

Intracellular localization of TAAR1

A major obstacle for careful pharmacological characterization of TAAR1was its extremely poor extracellular plasma membrane expression in model cell systems. In transfected cells, Bunzow et al (2001) had reported that an M1 flag-tagged rat TAAR1 had a largely intracellular distribution when stably expressed in HEK293 cells, in comparison to the predominant extracellular plasma membrane expression of anM1 flag-tagged human D1 dopamine receptor. The distribution of rat TAAR1 in the HEK293 cells appeared as intracellular puncta, in marked contrast to the localization of the human D1 dopamine receptor at the plasma membrane. Accordingly, TAAR1 did not traffic to the extracellular plasma membrane efficiently, leading the authors to suggest that rat TAAR1 might function in an intracellular environment. This notion was supported by the fact that biogenic amines are synthesized in the cytoplasm of monoaminergic cells, and that these amines as well as exogenous amphetamines can be imported into the cytoplasm and/or vesicular lumen because they serve as substrates of plasma membrane and vesicular monoamine transporters. Cellular distribution of rhesus monkey TAAR1 was also assessed. Miller et al (2005) observed a largely intracellular distribution of an EGFP-rhesus monkey TAAR1 chimera transiently expressed in HEK-293 cells and proposed that access of agonists to the receptor may be facilitated by translocation to the cell interior. More recently, Xie et al (2008b) used biotinylation and Western blotting to demonstrate that TAAR1 protein was mainly associated with the total membrane fraction in transfected HEK293 cells, but mainly not with the cell surface membrane.

Several strategies were employed to promote membrane expression of TAAR1 for the purpose of facilitating pharmacological characterization. These included modifying

intracellular loops, developing human-rat chimeras (Lindemann and Hoener, 2005; Reese et al.,2007) and co-expressing human TAAR1 with rat $G_{\alpha s}$ (Wainscott et al., 2007). Barak et al (2008) reported that insertion of the nine-amino-acid proximal portion of the human β2-adrenergic receptor into the N terminus of TAAR1 could stabilizeit at the plasma membrane. These data provided evidence that glycosylation-stabilized expression of the receptor can occur in cells at levels sufficient for characterizing receptor biology and developing reliable in vitro cellular assays.

TAAR1 is expressed in brain monoaminergic systems

Borowsky et al (2001) had also demonstrated that human and mouse TAAR1 mRNA was localized in various central nervous system and peripheral tissues and most notably in dopaminergic and adrenergic brain nuclei. Similar observations were made by Xie et al (2007) for rhesus TAAR1 mRNA. TAAR1 mRNA and protein expression was detected in rhesus monkey brain regions, including various regions throughout the monoaminergic systems, and TAAR1 co-expression with the dopamine transporter was detected in a subset of dopamine neurons in both rhesus monkey and mouse substantia nigra(Xie et al, 2007). Lindemann et al (2008) examined TAAR1 distribution in brain indirectly in transgenic mice. These investigators generated a mouse in which the entire TAAR1 coding sequence was replaced by a reporter gene consisting of LacZ fused to an N-terminal nuclear localization signal (NLS), and used an NLS-tagged version of the LacZ reporter to determine the expression and distribution of TAAR1 in the mouse brain. Specific labeling of brain nuclei was observed in monoaminergic regions including the substantia nigra, dorsal raphe nucleus and ventral tegmental area, as well as the hypothalamus and preoptic area, amygdala, nucleus of the solitary tract, and the parahippocampal region (rhinal cortices) and subiculum(Lindemann et al, 2008).It is important to note here that while co-localization of TAAR1 and the dopamine transporter has been observed in a subset of mouse and rhesus monkey substantia nigra neurons, co-localization of TAAR1 with either the norepinephrine transporter or the serotonin transporter has as of yet not been directly visualized anatomically, although data generated in striatal and thalamic synaptosomes from mice and monkeys strongly suggests co-localization with TAAR1 in noradrenergic and serotonergic synaptic terminals (Xie et al. 2008a; Xie and Miller, 2008). Preliminary identification of norepinephrine transporter and TAAR1 co-localization has also been observed using chromogenic immunocytochemical staining in some neurons within rhesus monkey locus ceoruleus(Miller, unpublished).

Monoamine transporters enhance TAAR1 signaling in vitro

The observation that TAAR1 is expressed in brain monoaminergic systems and co-localizes with the dopamine transporter in some dopamine neurons in brain suggested that the dopamine transporter could serve as a conduit for intracellular entry of the trace amines and amphetamines, increasing accessibility of these substrates to act as agonists at intracellular TAAR1 receptors. This hypothesis was tested in experiments in which rhesus monkey TAAR1 and the human dopamine transporter were co-transfected into HEK293 cells. Under these conditions, the degree of TAAR1 activation in response to biogenic amines and amphetamines was enhanced in CRE-luciferase reporter assays (Miller et al., 2005). These findings were then extended to the norepinephrine transporter and the serotonin transporter, which also conferred profound enhancement effects on rhesus monkey TAAR1 signaling in vitro (Xie et al., 2007). All compounds tested were known to be substrates at monoamine transporters, in addition to having the ability to activate TAAR1 signaling. The enhancement was shown to be transport dependent, in that specific monoamine transporter inhibitors could block the enhanced portion of the response. In a more recent study, the TAAR1 agonist 3-iodothyronamine (Scanlan et al., 2004) was assessed in this model system and it

did not show enhanced signaling at TAAR1 in the presence of the dopamine transporter, presumably because it is not a substrate but rather a dopamine transporter blocker (Panas et al., 2010). Together, these data demonstrate that monoamine transporters serve as conduits for biogenic amines and amphetamine substrates to enter cells as well as enhancers of TAAR1 signaling.

The observation that monoamine transporters enhance TAAR1 signaling suggested that intracellular TAAR1 receptors may be capable of signaling or may redistribute to the cell membrane. To date, there is no evidence yet reported for agonist-induced TAAR1 cellular redistribution. Nevertheless, TAAR1 and monoamine transporters apparently share a special synergistic relationship in that many TAAR1 activators are also transporter substrates. The enhanced TAAR1 signaling that occurs in cells that co-express TAAR1 and monoamine transporters further suggested that TAAR1 may play an important role in modulating signaling cascades in monoaminergic cells. One can speculate that enhanced TAAR1 signaling could in some cases be aberrant cell signaling, such as in response to amphetamine, and that TAAR1 activation in the milieu of a monoaminergic cell under such conditions may preferentially affect that cell over other cells that express TAAR1 but not monoamine transporters or vice versa. In this regard, TAAR1 could be involved in cellular adaptations to amphetamine-like drugs of abuse related to addiction, or the effects of substrates such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) related to neurotoxicity, which predominantly affect monoaminergic brain regions and dopaminergic neurons, respectively. Also, the data suggest that the efficacy of different TAAR1 agonists is a function not only of their affinity for TAAR1 and their ability to activate second messenger signaling cascades, but also their efficiency as a substrate at specific monoamine transporters and, as is the case with the common biogenic amines, their ability to activate other cellular receptors which may attenuate or augment TAAR1-trigered cell signaling cascades(such as monoamine autoreceptors), which will be discussed below.

TAAR1 signaling alters monoamine transporter function

An important concept now emerging is that receptor-mediated signaling events in monoaminergic neurons are the principal drivers of the phosphorylation-dependent mechanisms that in turn affect monoamine transporter functional regulation (e.g., Gulley and Zahniser, 2003; Middleton et al., 2004; Chefer et al., 2005; Zapata et al., 2007; Zhu et al., 2007; Xie and Miller, 2009a; Xie and Miller, 2009b Reyes et al., 2009; Iceta et al., 2009; Hope et al., 2010; Bowton et al., 2010).Accordingly, the ability of monoamine transporters to enhance TAAR1 signaling raised the possibility that the enhanced signaling of TAAR1 could in turn alter functional aspects of monoamine transporters. Xie and Miller (2007) tested this hypothesis by measuring the effects of TAAR1 activation on dopamine transporter-mediated [^{3}H]dopamine transport in TAAR1 and dopamine transporter co-transfected HEK293 (TAAR1-DAT) cells treated with either dopamine or methamphetamine. This approach was challenging because there were no available specific TAAR1 antagonists or agonists available as tools. Because both dopamine and methamphetamine are TAAR1 agonists as well as dopamine transporter substrates, a comparison of TAAR1-DATtodopamine transporter transfected (DAT) cells was used to distinguish between competitive inhibitory effects and TAAR1-mediated non-competitive effects of dopamine and methamphetamine on [^{3}H]dopamine uptake. Using various concentrations of [^{3}H]dopamine, Xie and Miller (2007) demonstrated that TAAR1 activation caused a concentration-and time-dependent inhibition of [^{3}H]dopamine uptake by the dopamine transporter. TAAR1 activation by 10 nM dopamine was very weak, as measured using a CRE-luciferase assay(Xie et al., 2007). In accordance, TAAR1-DATand DAT cells did not display any differences in the time-dependent uptake of 10 nM [^{3}H]dopamine. Both 100 nM and 1 μM concentrations of dopamine were shown to robustly

activate TAAR1 in the CRE-Luciferase assay(Xie et al, 2007), and at100 nM a transient inhibition of [^{3}H]dopamine uptake was observed in time course assays in the TAAR1-DAT cells but not the DAT cells(Xie et al., 2008a). Similarly, 90 nM methamphetamine was shown to activate TAAR1 in the CRE-Luciferase assay(Xie and Miller, 2007). When added with 10 nM [^{3}H]dopamine (total concentration of substrate 100 nM), 90 nM methamphetamine was also able to induce a transient uptake inhibition after 3minin TAAR1-DAT cells but not in DAT cells(Xie and Miller, 2007).Also, the transient uptake inhibition could be blocked by either a protein kinase A (PKA) inhibitor or a protein kinase C (PKC) inhibitor. Accordingly, these data provided evidence that dopamine uptake by the dopamine transporter was affected by TAAR1 signaling in response to dopamine or methamphetamine in vitro.

Elevated levels of cAMP induced by TAAR1 activation may underlie the TAAR1-dependent effects on [^{3}H]dopamine uptake, because a similar uptake inhibition was observed in control experiments that substituted the D1 dopamine receptor for TAAR1(D1-DAT cells) and used dopamine as an agonist, or that used forskolin treatments at different time points to directly activate cAMP(Xie and Miller, 2007). Interestingly, the time delay for observing D1-mediated inhibition of 100 nM[^{3}H]dopamine uptake in D1-DAT cells was1 min less than the time delay for observing TAAR1-mediated inhibition of 100 nM[^{3}H]dopamine uptake in TAAR1-DAT cells, consistent with [^{3}H]dopamine having greater access to D1 than TAAR1. D1 largely resides on the extracellular membrane(Trogadis et al., 1995; Kliem et al., 2010), and so this observation suggested that TAAR1 was intracellularly sequestered to a greater extent than D1 when expressed in HEK293 cells. Based on these data, Xie and Miller (2007) speculated that the time delay for TAAR1-mediated uptake inhibition may reflect a time-dependent accumulation of substrate within the cells to reach a sufficient concentration to activate an intracellular pool of TAAR1 receptors; or alternatively, agonist-induced translocation of TAAR1 to the membrane. In support of the former hypothesis, both Bunzow et al (2001) and Miller et al (2005) had demonstrated that TAAR1 is largely intracellularly sequestered when expressed in vitro(using a M1 flag-tagged rat TAAR1 and EGFP-rhesus TAAR1 chimera, respectively), and Xie et al (2008b) later demonstrated that the rhesus monkeyTAAR1 receptor is associated with a cellular membrane fraction in vitro, but largely not with the extracellular plasma membrane, using cell fractionation techniques followed by biotinylation and Western blotting.

TAAR1–mediated effects are phosphorylation dependent

The effects of TAAR1 activation by dopamine or methamphetamine on [^{3}H]dopamine uptake may result from transport reversal and substrate efflux. To address this hypothesis, Xie and Miller (2007) assessed reverse transport by the dopamine transporter and showed that TAAR1 activation by methamphetamine or dopamine triggered[^{3}H]dopamine efflux in vitro. TAAR1-DAT and DAT cells were loaded with 20 nM [^{3}H]dopamine for 20 min at 25°C in serum free DMEM, washed and then treated with dopamine or methamphetamine. Under these conditions, DAT cells retained the loaded [^{3}H]dopamine when treated with cold dopamine or methamphetamine across a wide range of concentrations (1 nM to 10 μM). Under the same conditions, TAAR1-DAT cells exhibited a spontaneous and time-dependent loss of the loaded [^{3}H]dopamine over 30 min, and this loss was further augmented by dopamine or methamphetamine in a dose-dependent manner. Both the spontaneous and the substrate-induced [^{3}H]dopamine efflux was blocked by either the dopamine transporter blocker methylphenidate or by PKC inhibition by 10 μM Ro32-0432, providing evidence that the [^{3}H]dopamine loss was due to reverse transport by the dopamine transporter and raising the possibility that TAAR1 may couple to PKC(Xie and Miller, 2007). Also, there was a sharp increase at 3 to 5 min in the rate of dopamine and methamphetamine-induced

[^{3}H]dopamine efflux, parallel to the timing of the observed uptake inhibition, suggesting that the effects of TAAR1 activation on the inhibition of [^{3}H]dopamine uptake were at least in part due to the promotion of [^{3}H]dopamine efflux.

The finding by Xie and Miller (2007) that DAT cells loaded with 20 nM [^{3}H]dopamine did not show substrate-induced efflux over a range of concentrations of dopamine or methamphetamine was perplexing, because substrate-induced efflux by the dopamine transporter is a widely reported phenomenon. It must be noted, however, that studies have generally not been done wit h such low concentrations of [^{3}H]dopamine (e.g., Eshleman et al., 1994; Wilhelm et al., 2004).In a later study, Xie and Miller (2009a) resolved this issue by demonstrating that methamphetamine could induce [^{3}H]dopamine efflux in DAT cells or in TAAR1 knockout mouse striatal synaptosomes when the cells or synaptosomes were loaded with high concentrations of [^{3}H]dopamine (1 μM or higher), indicating that the direct effect of methamphetamine on the dopamine transporter to cause [^{3}H]dopamine efflux is dependent on the preloading concentration of [^{3}H]dopamine. But the efflux caused by methamphetamine under these conditions in DAT cells was not associated with either the PKA or the PKC phosphorylation pathways whereas it was blocked by methylphenidate.

Further studies showed that TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine efflux occurred at both lower and higher (1 μM) loading concentrations of [^{3}H]dopamine in TAAR1-DAT cells or in wild type mouse striatal synaptosomes, which could be blocked not only by methylphenidate but also by the PKC inhibitor Ro32-0432(Xie and Miller, 2009a). At the higher loading concentration, there was a greater amount of efflux observed in the presence of TAAR1(in TAAR1-DAT cells or wild type mouse striatal synaptosomes) than in its absence (in DAT cells or TAAR1 knockout mouse striatal synaptosomes), and it was only this relative greater amount of efflux that was sensitive to PKC inhibition. Accordingly, the TAAR1-mediated effects of methamphetamine on [^{3}H]dopamine efflux was dependent on PKC phosphorylation, whereas an observed TAAR1-independent, PKC-independent efflux occurs when cells or synaptosomes are loaded with high concentrations of [^{3}H]dopamine.

Several interesting hypotheses emerge from these data. First, whereas TAAR1-mediated inhibition of[^{3}H]dopamine uptake involves both the PKA and PKC pathways, the TAAR1-mediated effect on [^{3}H]dopamine efflux involves only the PKC pathway. This suggests that: 1) TAAR1signals through both the PKA and PKC pathways; 2) TAAR1-mediated inhibition of [^{3}H]dopamine uptake in time course experiments cannot be fully explained by a concurrent promotion of efflux; 3) the process of [^{3}H]dopamine efflux by the dopamine transporter is insensitive to the PKA pathway activation that is consequent to TAAR1 activation. This may or may not be the case with regard to regulation of efflux by the norepinephrine transporter or the serotonin transporter; 4) the loss of the loaded 20 nM [^{3}H]dopamine over 30 min in TAAR1-DAT cells but not DAT cells suggests that 20 nM dopamine could activate TAAR1 in the presence of the dopamine transporter. Data derived from CRE-luciferase assays indicated that this may be the case (Xie et al., 2008a). This activation was not maximal because additional dopamine or methamphetamine promoted additional efflux. Accordingly, the data indicate that functionally, TAAR1 is very sensitive to dopamine; and 5) the dopamine transporter apparently has a PKC-sensitive as well as a PKC-insensitive mechanism for dopamine efflux. The PKC-sensitive mechanism mediates dopamine efflux under conditions of relatively lower levels of dopamine (or substrate), whereas the PKC-independent mechanism initiates under higher levels of dopamine (or substrate). Also, the PKC-dependent mechanism is receptor-driven and can be initiated by TAAR1. This latter point should be emphasized, because it suggests that it is the summation of temporal signaling events by the full repertoire of receptors that exist on a dopamine neuron which drives dopamine transporter function, and that targeting receptors on these

neurons is a reasonable strategy for developing medications that modify monoamine transporter function and consequent monoamine levels in the brain.

The role of TAAR1 thus becomes particularly relevant to the effects of amphetamine-like psychostimulants and trace amines, because it is one of the few (if not the only) receptors known to be present that is sensitive to these compounds. Additionally, the role of TAAR1 thus becomes particularly relevant to the effects of the common biogenic amines, because unlike amphetamines and trace amines, these neurotransmitters bind to both TAAR1 as well as monoamine autoreceptors present in the same neurons, and these two receptor systems have different cellular signaling cascades that interact with each other.

Trace amines activate TAAR1 but not monoamine autoreceptors

Xie and Miller (2008) next evaluated the interaction of trace amines with TAAR1 and monoamine autoreceptors. First, they investigated whether monoamine autoreceptor activation could influence TAAR1 signaling. β-PEA, tyramine, tryptamine, and octopamine were shown to activate rhesus monkey TAAR1 in a dose-dependent manner in TAAR1-transfected cells in vitro(Xie and Miller, 2008). Co-expression of TAAR1 with a monoamine autoreceptor, either the dopamine D2shortisoform ($D2_s$), alpha adrenergic receptor 2A(α_{2A}), alpha adrenergic receptor 2B(α_{2B}), serotonin receptor 5-HT1A ($5HT_{1A}$) or serotonin receptor 5-HT1B ($5HT_{1B}$)had no effect on the activation of TAAR1 by 1 μM of any of these trace amines, as shown using the CRE-luciferase assay(Xie and Miller, 2008). Competition binding assays were also performed that utilized stable $D2_s$, α_{2A} or α_{2B}, or $5HT_{1A}$ or $5HT_{1B}$ cells and [^{3}H]dopamine, [^{3}H]norepinephrine or [^{3}H]serotonin, respectively, to assess whether the trace amines bound to the autoreceptors. The IC_{50} of dopamine for $D2_s$ was 0.19 ± 0.06 μM, norepinephrine for α_{2A} and α_{2B} was 0.025 ± 0.008 and 0.058 ± 0.018 μM, respectively, and serotonin for $5HT_{1A}$ and $5HT_{1B}$ was 0.048 ± 0.013 and 0.032 ± 0.011 μM, respectively(Xie and Miller, 2008).In contrast, the IC_{50} values of β-PEA, octopamine, tryptamine or tyramine at these receptors were all greater than 10 μM. Xie and Miller (2008) also demonstrated that 1 μM β-PEA inhibited the uptake of 10 nM [^{3}H]dopamine, 20 nM [^{3}H]norepinephrine and 20 nM [^{3}H]serotonin in TAAR1-DAT, TAAR1-NET and TAAR1-SERT cells, respectively, whereas uptake inhibition by 1 μM β-PEA did not occur in DAT, NET and SERT cells in the absence of TAAR1, nor in cells co-expressing the respective autoreceptors and monoamine transporter pairs(e.g., D2-DAT cells).These experiments indicated that trace amines activate TAAR1 but not monoamine autoreceptors.

In further studies, synaptosomes were prepared from striatum and thalamus of rhesus monkeys, wild type mice and TAAR1 knockout mice. These brain regions were selected on the basis of accumulated experimental data demonstrating that the striatum contained high levels of dopamine transporter and serotonin transporter protein and that thalamus contained high levels of norepinephrine transporter protein in rhesus monkey brain(Miller et al., 2001). In particular, thalamus was chosen because it yielded the highest norepinephrine transporter signal:noise ratio combined with high density in a screening of norepinephrine transporter binding sites in various regions of primate brain (Madras and Miller, unpublished), and on the basis of Positron Emission Tomography imaging data using [^{11}C]MeNER to develop baseline measures of norepinephrine transporter binding potential in the thalamus (Madras et al., 2006). In concordance with the in vitro data described above, studies using the synaptosomal preparations also revealed that 1 μM β-PEA inhibited the uptake of 10 nM [^{3}H]dopamine or 20 nM [^{3}H]serotonin in striatal synaptosomes derived from rhesus monkey or wild type mice, and it also inhibited the uptake of 20 nM [^{3}H]norepinephrine from thalamic synaptosomes prepared from the same brains. But in synaptosomes prepared from TAAR1 knockout mice, uptake inhibition by 1 μM β-PEA did not occur (due to the absence

of TAAR1). The same synaptosome preps were also used to investigate the influence of β-PEA on the efflux of [^{3}H]monoamine. In synaptosomes that were preloaded with [^{3}H]monoamine for 20 min and then washed and treated with 1 μM β-PEA, efflux of [^{3}H]dopamine or [^{3}H]serotonin from striatal synaptosomes as well as efflux of [^{3}H]norepinephrine from thalamic synaptosomes occurred in the rhesus monkey and wild type mouse synaptosomes but did not occur in the TAAR1 knockout synaptosomes (Xie and Miller, 2008). These experiments indicated that TAAR1 mediates the effects of β-PEA on uptake and efflux functions of the monoamine transporters and as such, this report represented the first description of a receptor-mediated mechanism by which trace amines can alter monoaminergic function in mammalian brain.

Common biogenic amines activate TAAR1 and monoamine autoreceptors

In a companion study, Xie et al (2008) evaluated the interaction of the common biogenic amines with TAAR1 and monoamine autoreceptors. Dopamine, norepinephrine and serotonin all promote CRE-luciferase expression in a TAAR1-dependent manner in CRE-luciferase assays in vitro (Xie et al., 2007; Xie et al., 2008a), indicative of elevations in cAMP. Accordingly, these common biogenic amines serve as agonists at rhesus monkey TAAR1. But unlike the trace amines, co-expression of TAAR1 with either $D2_s$, α_{2A} or α_{2B}, or $5HT_{1A}$ or $5HT_{1B}$ attenuated the CRE-luciferase expression induced by 1 μM of dopamine, norepinephrine or serotonin, respectively. The attenuation was completely blocked by a specific monoamine autoreceptor antagonist. For example, in D2s-TAAR1 cells, dopamine treatment resulted in very little signal but if the D2 antagonist raclopride was also present, these cells responded identically to TAAR1 cells. In experiments analogous to those which assessed β-PEA (Xie and Miller, 2008) and methamphetamine (Xie and Miller 2009a), activation of TAAR1 by the common biogenic amines was shown to result in uptake inhibition and promotion of efflux of [^{3}H]dopamine in TAAR1-DAT cells, [^{3}H]norepinephrine in TAAR1-NET cells and [^{3}H]serotonin in TAAR1-SERT cells in vitro, and these effects did not occur in the absence of TAAR1 in DAT, NET or SERT cells, respectively (Xie et al, 2008a). It was also shown that the common biogenic amines could cause an enhancement of uptake via activation of $D2_s$ in $D2_s$-DATcells, α_{2A} in α_{2A}-NETor $5HT_{1B}$ in $5HT_{1B}$-SERT cells, respectively(Xie et al, 2008a). Accordingly, the common biogenic amines could alter the kinetics of DAT, NET, and SERT via interaction with eitherTAAR1ormonoamine autoreceptors in vitro, and the receptor signaling cascades triggered by TAAR1 and the monoamine autoreceptors could counteract each other. Whereas TAAR1-transfected cells responded similarly to trace amines, common biogenic amines and amphetamines with regard to CRE-luciferase responses, and whereas TAAR1 signaling in turn altered the uptake and efflux properties of co-transfected monoamine transporters similarly in response to trace amines, common biogenic amines and amphetamines in vitro, findings in brain synaptosomes diverged for the common biogenic amines. In monkey and wild-type mouse brain synaptosomes, the common biogenic amines inhibited uptake only when the monoamine autoreceptors were blocked by selective monoamine autoreceptor inhibitors, and under the same condition, such effects of the common biogenic amines on [^{3}H]monoamine uptake did not occur inTAAR1 knockout mouse synaptosomes. When the autoreceptors were blocked, norepinephrine and serotonin promoted efflux of [^{3}H]norepinephrine and [^{3}H]serotonin from thalamic and striatal synaptosomes, respectively. Butin TAAR1 knockout mouse synaptosomes, norepinephrine and serotonin did not promote efflux(Xie and Miller, 2008a). Interestingly, dopamine induced [^{3}H]dopamine efflux in striatal synaptosomes of monkeys, wild-type mice, and alsoTAAR1 knockout mice in the absence of D2 blockade, which may suggest that, in addition to TAAR1, dopamine may also interact with another dopamine-sensitive receptor to trigger [^{3}H]dopamine efflux independent of TAAR1. Alternatively, there may be some adaptive compensatory changes in the dopamine system of the TAAR1 knockout mice. This

concept is supported by the observation that these mice have a 262% increase in the proportion of striatal high-affinity D2 receptors (Wolinsky et al., 2006).

It is notable that the synaptosomal preparations (Xie et al., 2008a; Xie and Miller, 2008; Xie and Miller, 2009a) were apparently discrete monoaminergic synapses. For example, whereas the D2 receptor inhibitor raclopride altered the dopamine effect on [^{3}H]dopamine uptake in monkey and wild-type mouse striatal synaptosomes, substitution of raclopride with the 5-HT_1 receptor inhibitor methiothepin did not change the dopamine effect(Xie and Miller, 2008a). In the same striatal synaptosomal preparations, methiothepin but not raclopride altered the serotonin effect on [^{3}H]serotonin uptake(Xie and Miller, 2008a).

Accordingly, these findings along with other evidence that TAAR1 is co-expressed with monoamine transporters in brain monoaminergic neurons (Borowsky et al., 2001; Miller et al., 2005; Xie etal., 2007; Xie and Miller, 2009b; Lindemann et al., 2008) indicate thatTAAR1 is present in monoaminergic neuronal terminals, that is a receptor for common biogenic amines, and that it functions as a presynaptic modulator of monoamine transporter activity in brain. The data also reveal that TAAR1 plays an important role as a monoamine autoreceptor in presynaptic terminals, functioning in coordination with the classic monoamine autoreceptors. In this regard, clinically relevant drugs that target the classic monoamine autoreceptors may cause effects in brain via creating an imbalance in the coordinated signaling of TAAR1 and monoamine autoreceptors in response to endogenous biogenic amines. Furthermore, the data reveal a mechanism by which trace amines and amphetamines can affect presynaptic neuronal function differently from the common biogenic amines, in that these compounds do not activate the monoamine autoreceptors but are potent activators of TAAR1. In this regard it should also be noted that micromolar concentrations of trace amines and amphetamine have been shown to reduce mouse D2 receptor-activated G protein-gated inward rectifier K+ channel (GIRK) currents through a mechanism that is independent of TAAR1 (Ledonne et al, 2010).

The TAAR1 receptor responds to wide spectrum of compounds, including both the common biogenic amines and trace amines (as well as many others; e.g., see Bunzow et al., 2001). As its name implies, the receptor is activated by the trace amines (e.g., β-PEA, tyramine) at nanomolar concentrations(reviewed in Berry, 2004). In functional assays (in vitro and in brain synaptosomes), sub-nanomolar concentrations of the trace amines do not elicit receptor activation as indicated by the CRE-luciferase assay or by monitoringtheTAAR1-mediated effect on monoamine transporter function (Xie et al., 2007; Xie and Miller, 2008). It is the case that in the presence of an MAOB inhibitor, trace amines escape metabolism and reach levels suitable for TAAR1to be activated, but perhaps not in the absence of this pharmacological intervention (Bergman et al., 2001). Whether or not this is the case, what is apparent is that common biogenic amines, and in particular dopamine, activate rhesus monkey, tamarin monkey and mouse TAAR1 and that this activation by dopamine results in measurable and reproducible downstream effects that include effects on dopamine transporter kinetic function (Xie and Miller, 2007; Xie et al., 2008a). In membranes prepared from cells transiently transfected with human TAAR1 and rat $G_{\alpha s}$, dopamine has a reported Ki value of ~420 nM for displacing [^{3}H]tyramine (20 nM) (Borowsky et al., 2001). Dopamine, unlike what has to date been observed for trace amines, does achieve concentration in brain that exceed its Ki value (exceed 1 μM)in striatum(Hocevar et al., 2006; Njagi et al., 2010), and so it is reasonable to think of dopamine as an endogenous ligand at TAAR1.It is principally for these reasons that a newly-proposed nomenclature designated by the International Union of Pharmacology (IUPHAR) for TAAR1 as TA_1, based on trace amines being the cognate endogenous ligands for the receptor, has not been adopted in this overview nor by this author generally(Maguire et al, 2009).

TAAR1-mediated dopamine transporter internalization

Xie and Miller (2009a) next provided the first data which indicated that TAAR1 signaling can function as a triggering mechanism for the phosphorylation-driven cellular events that hallmark dopamine transporter internalization. Methamphetamine (1 μM) reduced cell surface levels of the dopamine transporter(by about 70%) in TAAR1-DAT cells in vitro and in rhesus monkey and wild-type mouse striatal synaptosomes ex vivo, whereas it did not internalize the dopamine transporter in DAT cells and had only a weak effect in TAAR1 knockout mouse striatal synaptosomes(Xie and Miller, 2009a). TAAR1-dependent dopamine transporter internalization induced by methamphetamine was blocked by the PKC inhibitor Ro32-0432but not by the PKA inhibitor H89(Xie and Miller, 2009a). The internalization may result in a lower transport capacity of the dopamine transporter, which is consistent with the observed TAAR1-mediated inhibition of [^{3}H]dopamine uptake discussed above, whereas [^{3}H]dopamine efflux that occurs at a similar time with the observed dopamine transporter internalization may be mediated by a population of dopamine transporter molecules that remain at the cell surface.

TAAR1 knockout mice show greater sensitivity to amphetamine and higher dopaminergic tone

Two reports demonstrated an enhanced sensitivity of TAAR1 knockout mice to amphetamine and an amphetamine-induced increase in the release of biogenic amines in the TAAR1 knockout mouse striatum (Wolinsky et al., 2007; Lindemann et al., 2008). Wolinsky et al (2007) observed that TAAR1 knockout mice showed an enhanced sensitivity to the psychomotor-stimulating effect of 1 mg/kg *d*-amphetamine, which was temporally correlated with significantly larger increases in the release of both dopamine and norepinephrine in the dorsal striatum. Wolinsky et al (2007) also reported a 262% increase in the proportion of striatal high-affinity D2 receptors. In a different mouse line, Lindemann et al (2008) reported that TAAR1 knockout mice show an increased locomotive response to *d*-amphetamine after a single application of either 1 mg/kg or 2.5 mg/kg. The increased locomotion correlated with a two-to three-fold increase in extracellular dopamine, norepinephrine and serotonin levels in the TAAR1 knockout mice as compared to wild type mice. This pharmacologically-induced phenotypic difference was observed in the absence of any physical or behavioral phenotypic differences between wild type and TAAR1 knockout mice, including basal locomotor activity. Indeed, the only other phenotypic difference reported is that TAAR1 knockout mice display a deficit in prepulse inhibition (Wolinsky et al., 2007).

In further studies, electrophysiological analysis of dopaminergic neurons in the ventral tegmental area of TAAR1 knockout and wild type mice revealed that the spontaneous firing rate of dopaminergic neurons was 8.6-fold higher in the TAAR1 knockout mice, and that activation of TAAR1 by 10 μM *p*-tyramine decreased the firing rate of dopaminergic neurons in wild type mice but not in TAAR1 knockout mice(Lindemann et al., 2008). Trace amines such as *p*-tyramine are known to reduce the firing frequency of dopamine neurons (Geracitano et al., 2004; Lindemann et al., 2008;Rodriguez et al., 1995).Bradaia et al (2009) found that TAAR1 not only tonically activated inwardly rectifying potassium channels, but also that acute blockade of TAAR1 increased the affinity of dopamine at D2 receptors. Incubation of ventral tegmental area brain slices with the first TAAR1 antagonist to be developed, EPPTB, was shown to increase the spontaneous firing rate of dopamine neurons above the basal level, consistent with the increased firing rate of dopamine neurons observed in the TAAR1 knockout mice. It was also shown that in Xenopus oocytes, TAAR1 directly activated Kir3 channels via a PTX-insensitive G-protein, most likely G_s. Together, these findings suggest that TAAR1 is tonically active under physiological conditions, either due to

the presence of an ambient ligand or because of constitutive receptor activity (Lindemann et al., 2008; Bradaia et al., 2009).The data support the hypothesis that TAAR1 inhibits locomotor activity via a down modulation of dopamine neurotransmission (Lindemann et al., 2008) and that the overruling effect of blocking TAAR1 is a net increase in the firing rate of DA neurons (Bradaia et al., 2009). However, a more recent study by Sotnikova et al (2010) reports that the major extracellular metabolite of dopamine, 3-methoxytyramine (3-MT), which is an agonist at rat TAAR1 (Bunzow et al., 2001), can induce mild hyperactivity in normal mice and a complex set of abnormal involuntary movements in normal mice acutely depleted of dopamine, and that these effects were attenuated in TAAR1 knockout mice. These data suggest that TAAR1 activation may stimulate locomotor activity. Collectively, the data illustrate a complexity of TAAR1 neurobiology that is still not fully understood.

Future directions and a working model

Because neuroadaptive changes such as a dramatic increase of high-affinity D2 receptors occur in the TAAR1 knockout mice (Wolinsky et al., 2007), it is possible that their enhanced sensitivity to amphetamine may not be the direct consequence of the TAAR1 deficit but rather may be due to developmental adaptations. However, Xie et al (2007; 2009b) have observed neurons scattered in the midbrain of both rhesus monkey and mouse that stained positive for TAAR1 but negative for the dopamine transporter. These investigators have hypothesized that TAAR1-positive neurons may be presynaptic to dopamine neurons and form inhibitory synapses on dopamine neurons, capable of influencing dopamine release (exocytosis) and dopamine transporter function (uptake or efflux) via changes in signal transduction in dopamine neurons. If this were the case, then the deprivation of this TAAR1-mediated inhibition on dopamine neurons in response to amphetamine in TAAR1 knockout mice could result in their hypersensitivity to amphetamines. Future studies need to examine more thoroughly the distribution of TAAR1 in the brain and the role of TAAR1 in interneuronal communication as a first step in assessing the validity of this hypothesis.

Our understanding of TAAR1 in brain monoaminergic systems and throughout the rest of the brain generally is still at a very early stage. The functional studies discussed here have focused on the role of TAAR1 in monoaminergic systems and its interaction with monoamine transporters, particularly the dopamine transporter. Nevertheless, these studies and those that describe an enhanced response to amphetamine in TAAR1 knockout mice can be synthesized into a working model which can facilitate further investigations and may also help to summarize herein the complexities that have emerged so far in the research(Figure 1).Also shown here is a previously unpublished Confocal image of TAAR1 and DAT double label immunofluorescence which shows TAAR1-expressing neurons directly adjacent to dopamine neurons in the rhesus monkey substantia nigra(Figure 2).

Collectively, the functional studies on the role of TAAR1 reviewed here indicate that TAAR1 is implicated in the functional regulation of the dopamine system, and evoke the concept that TAAR1 is involved in the homeostatic regulation of dopamine and the biochemical cascades triggered by amphetamine-like psychostimulants. These studies suggest a new avenue of approach for medications development to treat neuropsychiatric disorders and psychostimulant addictions. Monoamine transporters are frontline targets of therapeutics that are prescribed clinically for the treatment of a spectrum of neuropsychiatric disorders, and are also major targets of psychostimulant drugs of abuse. As a modulator of brain monoaminergic systems and a direct target of amphetamine-like psychostimulants, research on TAAR1 will likely promote the development of a new generation of therapeutics

that modulate monoamine levels in brain in a manner different from direct binding and blockade of monoamine transporters.

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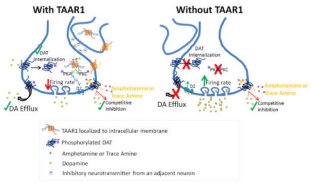

Figure 1. A hypothetical model that summarizes TAAR1 agonist effects at the dopamine neuronal terminal incorporating in vitro, ex vivo and in vivo data

Left: Depiction of a dopaminergic synaptic terminal that expresses TAAR1. TAAR1 activation triggers cellular signaling events including activation of PKA and PKC that lead to phosphorylation-dependent dopamine efflux via the dopamine transporter and dopamine transporter internalization. In addition, TAAR1 agonists (such as amphetamine, methamphetamine or trace amine) compete with dopamine for reuptake (competitive inhibition). These processes raise extracellular dopamine levels. Bradaia et al (2009) has reported that the TAAR1 antagonist, EPPTB, increases the firing frequency of dopamine neurons, suggesting that TAAR1 either exhibits constitutive activity or is tonically activated by ambient levels of endogenous agonist. These investigators also showed a reduction in firing frequency of dopamine neurons in response to the TAAR1 agonist tyramine. In mouse substantia nigra, Xie et al (2007) observed frequent TAAR1-expressing neurons in the midst of DAT-positive neurons and processes that did not coexpress DAT but that were in approximation to DAT neurons (a previously unpublished image illustrating this proximity is shown in Figure 2). Accordingly, the model presented predicts that TAAR1 agonists decrease the firing frequency of dopamine neurons by activating TAAR1 in adjacent inhibitory neurons, while also increasing extracellular levels of dopamine via both competitive inhibition of reuptake of dopamine as well as by non-competitive, TAAR1-mediated uptake inhibition and promotion of efflux. It is notable that the increased levels of extracellular dopamine may not be highly localized to the dopaminergic synapse, in that the location of dopamine transporters spans the neuron throughout its entire somatodendritic and axonal domains and at terminals resides outside of synaptic active zones, whereas dopamine is released from the somata of substantia nigra neurons by exocytosis in response to neuronal electrical activity (Ciliax et al. 1999; Jaffe et al. 1998). Also, these non-competitive effects are PKA- and PKC-dependent and are attenuated by D2 receptor activation. ***Right:*** Depiction of a dopaminergic terminal in a TAAR1 knockout mouse. In the absence of TAAR1, agonists such as amphetamine and methamphetamine do not trigger phosphorylation-dependent dopamine efflux via the dopamine transporter nor dopamine transporter internalization, yet the competitive inhibition of dopamine reuptake (which is TAAR1-independent) by amphetamine still occurs. In the absence of TAAR1, TAAR1 knockout mice show a greater locomotor response to amphetamine and release more dopamine (and norepinephrine) in response to amphetamine than do WT mice. As TAAR1 activation decreases the firing frequency of dopamine neurons, TAAR1 knockout mice may have a higher firing frequency which is not decreased in response to amphetamine. The collective effects of these processes may result in the higher synaptic dopamine levels induced by amphetamine in TAAR1 knockout mice, and also explain the enhanced behavioral response to amphetamine.

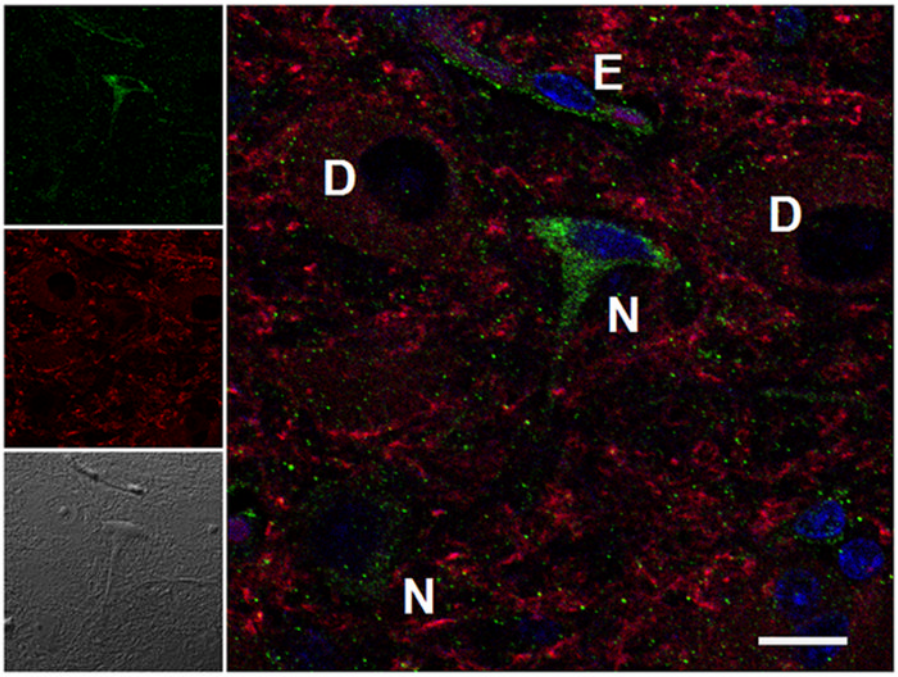

Figure 2. A Confocal image showing TAAR1-positive non-dopaminergic neurons adjacent to DAT-positive dopaminergic neurons in substantia nigra of an adult rhesus monkey

In the double-label immunofluorescent image (right), neurons are present that express either TAAR1 (green) or DAT (red), but not both. TAAR1 staining is observed in two neurons (N) with apparent intracellular localization, as well as in an epithelial cell (E) where it appears to be localized to the extracellular plasma membrane. Note the proximity of TAAR1-positive neurons to two dopamine neurons (D). Nuclei (blue); red blood cells in endothelial cell (purple). Smaller images of individual channels, including TAAR1 (green), DAT (red), and differential interference contract (DIC), are shown on the left side of the larger composite panel. Micrometer bar = 20 microns. Methods can be found in Xie et al., 2007.