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Salvinorin A regulates dopamine transporter function via a kappa opioid receptor and ERK1/2-dependent mechanism

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ABSTRACT

Salvinorin A (SalA), a selective κ-opioid receptor (KOR) agonist, produces dysphoria and pro-depressant like effects. These actions have been attributed to inhibition of striatal dopamine release. The dopamine transporter (DAT) regulates dopamine transmission via uptake of released neurotransmitter. KORs are apposed to DAT in dopamine nerve terminals suggesting an additional target by which SalA modulates dopamine transmission. SalA produced a concentration-dependent, nor-binaltorphimine (BNI)- and pertussis toxin-sensitive increase of ASP^+ accumulation in EM4 cells coexpressing myc-KOR and YFP-DAT, using live cell imaging and the fluorescent monoamine transporter substrate, trans 4-(4-(dimethylamino)-styryl)-N-methylpyridinium) (ASP^+). Other KOR agonists also increased DAT activity that was abolished by BNI pretreatment. While SalA increased DAT activity, SalA treatment decreased serotonin transporter (SERT) activity and had no effect on norepinephrine transporter (NET) activity. In striatum, SalA increased the V_{max} for DAT mediated DA transport and DAT surface expression. SalA up-regulation of DAT function is mediated by KOR activation and the KOR-linked extracellular signal regulated kinase-½ (ERK1/2) pathway. Co-immunoprecipitation and BRET studies revealed that DAT and KOR exist in a complex. In live cells, DAT and KOR exhibited robust FRET signals under basal conditions. SalA exposure caused a rapid and significant increase of the FRET signal. This suggests that the formation of KOR and DAT complexes is promoted in response to KOR activation. Together, these data suggest that enhanced DA transport and decreased DA release resulting in decreased dopamine signalling may contribute to the dysphoric and pro-depressant like effects of SalA and other KOR agonists.

Abbreviations: ASP^+, trans 4-(4-(dimethylamino)-styryl)-N-methylpyridinium); BNI, nor-binaltorphimine; BRET, Biolumiescence resonance energy transfer; DAT, dopamine transporter; ERK1/2, Extracellular signal-regulated kinases ½; FRET, Fluorescence resonance energy transfer; GPCR, G-protein coupled receptor; KOR, *kappa* Opioid receptor; SalA, Salvinorin A.

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1. Introduction

Salvinorin A (SalA) is a naturally occurring κ-opioid receptor (KOR) agonist that produces psychotomimesis, dysphoria and prodepressant like effects (Vortherms and Roth, 2006). Despite a surge in its recreational use (Griffin et al., 2008), the neural correlates mediating these effects are unknown. Decreased dopamine signalling in the dorsal and ventral striatum is implicated in the induction of dysphoria and the pathogenesis of depression (Mizrahi et al., 2007). KOR agonists decrease dopamine concentrations in these regions; an effect attributed to inhibition of dopamine release (Shippenberg et al., 2007; Spanagel et al., 1990).

The dopamine transporter (DAT) rapidly clears the dopamine released into the extracellular space and the activity of DAT is the determinant of dopamine signalling (Amara and Kuhar, 1993). It is largely evident that DAT activity is rapidly altered in response to activation and or inhibition of several protein kinases and protein phosphatase PP1/PP2Ac. For example, activation of PKC as well as inhibition of ERK1/2, PI-3 kinase, CaMKII, Cdk5 and tyrosine kinase rapidly decrease DA uptake (Carvelli et al., 2002; Doolen and Zahniser, 2001; Fog et al., 2006; Moron et al., 2003; Price et al., 2009; Zhang et al., 1997). In addition, while activation of D2, D3 DA receptors ($D_{2S}R$, D_3R), TrkB and insulin receptors stimulates DA uptake, activation of NK1R or mGluR5 reduces DA uptake (Bolan et al., 2007; Garcia et al., 2005; Granas et al., 2003; Hoover et al., 2007; Lee et al., 2007; Mayfield and Zahniser, 2001; Page et al., 2001; Zapata et al., 2007). Thus, G-protein coupled receptor (GPCR) and non-GPCR receptor regulation of DAT has been well documented (reviewed and references therein (Ramamoorthy et al., 2011)). The contributions of regulated DAT phosphorylation (Khoshbouei et al., 2004), ubiquitylation (Miranda et al., 2005), palmitoylation (Foster and Vaughan, 2011), glycosylation (Li et al., 2004), protein–protein interaction (reviewed (Eriksen et al., 2010)-therein references) and lipid-raft distribution (Adkins et al., 2007) have been demonstrated in regulating DAT-trafficking, DAT-mediated DA-efflux and degradation. Furthermore, DAT-substrates and inhibitors can also influence kinase/phosphatase mediated DAT regulation (Melikian, 2004; Pramod et al., 2013; Ramamoorthy et al., 2011; Vaughan and Foster, 2013).

KOR is apposed to DAT in striatal dopamine axons and varicosities (Svingos et al., 2001). These findings indicate that KOR is strategically located to regulate dopamine uptake and that DAT may be a target upon which SalA acts to modulate dopamine transmission and behaviour. However, our knowledge of the SalA mediated KOR-signalling mechanisms that contribute to DAT regulation is incomplete. The present studies, conducted in heterologous expression systems and native tissue, examined whether SalA modulates DAT function and the intracellular mechanisms mediating this effect.

2. Materials and methods

2.1. Live cell imaging to quantify DAT function

Experiments were conducted in HEK-293 (HEK) and EM4 cells, a HEK cell line expressing a macrophage scavenger receptor to increase adherence to tissue culture plastic. Cells were maintained in DMEM/Ham's F-12 medium (50:50; Mediatech Inc., Herndon, VA) supplemented with 10% FBS and grown in a humidified atmosphere (37 °C and 5% CO_2). Cells were transfected with myc-rat KOR (KOR; 0.1 μg) and either 0.3 μg of YFP-human DAT (DAT), eGFP-rat DAT (rDAT), FLAG-human DAT (FLAG-DAT) or GFP-human norepinephrine transporter (NET), or human serotonin transporter (hSERT) 24 h after plating, using Lipofectamine™ LTX (Invitrogen, Carlsbad, CA). Experiments were performed 48 h later (cell confluency: 70–80%). Addition of these tags does not alter the trafficking, protein localization or function (Jordan and Devi, 1999; Zapata et al., 2007).

Time-resolved quantification of DAT function in single cells was achieved using the fluorescent, high affinity monoamine transporter substrate 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (ASP^+) (Schwartz et al., 2003). ASP^+ is a sensitive probe for monitoring monoamine transporter function (Bolan et al., 2007; Schwartz et al., 2003; Zapata et al., 2007). ASP^+ accumulation is linear for 10 min after ASP^+ addition, inhibited by substrates and dependent on temperature and extracellular NaCl concentrations. Immediately before experiments, media was removed and cells washed in Krebs-Ringer/HEPES medium (KRH in mm: 130 NaCl, 1.3 KCl, 2.2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 HEPES, and 1.8 g/liter glucose, pH 7.4). Fresh KRH was added, and the culture dish was mounted on an UltraVIEW™ LCI spinning-disk (PerkinElmer Life Sciences (Grand Island, NY)) or Olympus FV1000 (Olympus, Tokyo, Japan) confocal microscope (60× water objective lens). A within cell design was used to assess the effects of graded SalA concentrations (0.1–10 μm), and to determine ASP^+ uptake kinetics. The microscope was focused on a cell monolayer and background auto-fluorescence determined by collecting an image immediately prior to replacing buffer with that containing ASP^+ (10 μm). SalA, U69,593 or U50,488 or vehicle was added 5 min later. The slope of ASP^+ accumulation was determined before and after addition. Images were collected every 20 s for 10 min to capture YFP or GFP (excitation, 488 nm; emission, 525–575 nm) and ASP^+ fluorescence (excitation, 488 nm; emission, 607–652 nm). To determine ASP^+ uptake kinetics, a range of ASP^+ concentrations (0–16 μM) were used. The slope of uptake phase was determined following background subtraction (cells not expressing DAT) and normalization to cell surface expression of either DAT or SERT. For studies assessing the effects of the selective KOR antagonist, norbinaltorphimine (BNI: 1 μM; 10 min), pertussis toxin (PTX: 100 ng/ml; 16–24 h) or the selective extracellular signal regulated kinase 1/2 (ERK) inhibitor, PD98059 (10 μM; 15 min) or p38 MAP kinase inhibitor SB203580 (3 μM; 5 min) (Tocris; Minneapolis, MN) or dopamine D_2 receptor antagonist L-741,626 (10 μM; 5 min) (Tocris; Minneapolis, MN) on SalA-evoked alterations of ASP^+ uptake, cells were incubated with drug for the indicated times and the slope of ASP^+ accumulation quantified as above. Drug concentrations were chosen based on reported effective concentrations (Alessi et al., 1995; Bolan et al., 2007; Dalman and O'Malley, 1999; Grilli et al., 2009; Zapata et al., 2007). Fluorescent images were processed using Velocity (PerkinElmer Life Sciences) and NIH ImageJ (version 1.32) software. Within cell fluorescent accumulation, defined by GFP or YFP plasma membrane fluorescence, was measured as average pixel intensity of time-resolved images. Data are expressed as arbitrary fluorescence units (AFU) or percent change in ASP^+ uptake rate after drug addition. Typically 30–100 cells from three separate transfections were used.

2.2. Quantification of striatal DAT function

All Procedures with rodents were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide (NIH Publication No. 8023, revised 1978) for the Care and Use of Laboratory Animals and the Victoria University of Wellington Animal Ethics Committee. Rats were maintained in a temperature and humidity controlled room on a 12:12 h light/dark cycle. Food and water were supplied *ad* libitum. All efforts and care were taken to minimize animal suffering and to reduce the number of animals used. As alternatives to brain tissues, cell culture models were utilized.

2.2.1. Rotating disk electrode (RDE) voltammetry

RDE was used to determine the initial velocity of dopamine clearance in minces of the striatal tissue of rats as previously described using an electrode rotation rate of 4000 rpm and an applied potential of +450 mV versus Ag/AgCl reference electrode (Thompson et al., 2000). Voltage output was monitored until stable baselines were obtained (≅10 min). SalA or U50,488 (3 μl; final cell concentration: 10 nM) or an equivalent volume of vehicle was added to the electrochemical cell 4–5 min prior to addition of dopamine (6 μl; final concentration: 2 μM). PD98050 (3 μl; final concentration: 10 μM) was added to the cell, followed 10 min later by SalA. The resultant signals were detected as changes in voltage output versus time using electrochemical detection. The initial rate of signal decay after dopamine addition was calculated for 10 s. Rates of nonspecific signal decay, defined as signal decay in the absence of tissue at the end of each experimental day, were subtracted from that in the presence of tissue to calculate initial velocity of DA clearance (pmoles/s/g wet weight tissue). DA-clearance in the presence of GBR12909 was subtracted from DA clearance in absence of GBR12909 to derive DAT-mediated DA clearance.

2.2.2. [^{3}H]DA uptake assay

Synaptosomes from striatum were prepared and [^{3}H]DA uptake was measured as described previously (Tejeda et al., 2013). Briefly, rats were rapidly decapitated, and striatal regions were dissected and collected in 10 volumes (wt/vol) of cold 0.32 M sucrose. The tissue was immediately homogenized using a Teflon-glass homogenizer and centrifuged at 1000 × *g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 12,000 × *g* for 20 min and the pellet was washed by resuspending in 0.32 M sucrose. The synaptosomal preparation was used immediately for experiments. Protein concentration was determined by DC protein assay (BioRad) using bovine serum albumin as standard. Striatal synaptosomes (40 μg) were incubated in a total volume of 0.5 ml of Krebs-Ringer-HEPES (KRH) buffer consisting of 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$ 10 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, 10 mM D-glucose, pH 7.4 containing 0.1 mM ascorbic acid, and 0.1 mM pargyline in the presence of SalA (10 μM) or appropriate vehicle at 37 °C for 5 min. Uptake was initiated by the addition of 10 nM [^{3}H]DA (78 Ci/mmol-