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Research Paper

Strength of cholinergic tone dictates the polarity of dopamine D2 receptor modulation of striatal cholinergic interneuron excitability in DYT1 dystonia

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ABSTRACT

Balance between cholinergic and dopaminergic signaling is central to striatal control of movement and cognition. In dystonia, a common disorder of movement, anticholinergic therapy is often beneficial. This observation suggests there is a pathological increase in cholinergic tone, yet direct confirmation is lacking. In DYT1, an early-onset genetic form of dystonia caused by a mutation in the protein torsinA (TorA), the suspected heightened cholinergic tone is commonly attributed to faulty dopamine D2 receptor (D2R) signaling where D2R agonists cause excitation of striatal cholinergic interneurons (ChIs), rather than the normal inhibition of firing observed in wild-type animals, an effect known as "paradoxical excitation". Here, we provide for the first time direct measurement of elevated striatal extracellular acetylcholine (ACh) in a knock-in mouse model of human DYT1 dystonia ($TorA^{\Delta E/+}$ mice), confirming a striatal hypercholinergic state. We hypothesized that this elevated extracellular ACh might cause chronic over-activation of muscarinic acetylcholine receptors (mAChRs) and disrupt normal D2R function due to their shared coupling to $G_{i/o}$-proteins. We tested this concept *in vitro* first using a broad-spectrum mAChR antagonist, and then using a M2/M4 mAChR selective antagonist to specifically target mAChRs expressed by ChIs. Remarkably, we found that mAChR inhibition reverses the D2R-mediated paradoxical excitation of ChIs recorded in slices from $TorA^{\Delta E/+}$ mice to a typical inhibitory response. Furthermore, we recapitulated the paradoxical D2R excitation of ChIs in striatal slices from wild-type mice within minutes by simply increasing cholinergic tone through pharmacological inhibition of acetylcholinesterase (AChE) or by prolonged agonist activation of mAChRs. Collectively, these results show that enhanced mAChR tone itself is sufficient to rapidly reverse the polarity of D2R regulation of ChI excitability, correcting the previous notion that the D2R mediated paradoxical ChI excitation causes the hypercholinergic state in dystonia. Further, using a combination of genetic and pharmacological approaches, we found evidence that this switch in D2R polarity results from a change in coupling from the preferred $G_{i/o}$ pathway to non-canonical β-arrestin signaling. These results highlight the need to fully understand how the mutation in TorA leads to pathologically heightened extracellular ACh. Furthermore the discovery of this novel ACh-dopamine interaction and the participation of β-arrestin in regulation of cholinergic interneurons is likely important for other basal ganglia disorders characterized by perturbation of ACh-dopamine balance, including Parkinson and Huntington diseases, l-DOPA-induced dyskinesia and schizophrenia.

1. Introduction

Striatal control of motor and cognitive functions relies on proper balance between cholinergic and dopaminergic modulation of synaptic circuits. This balance is ensured by a complex, close interaction between cholinergic interneurons (ChIs) and midbrain dopaminergic neurons (Stoof et al., 1992a; Pisani et al., 2007; Aosaki et al., 2010). These two neuron populations exhibit coincident physiological changes in their activity during behavior (Morris et al., 2004; Joshua et al., 2008) and exert

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ChI, cholinergic interneurons; D2R, dopamine D2 receptors; mAChR, muscarinic receptors; Oxo, oxotremorine; Quin, quinpirole; Neo, neostigmine; TorA, torsinA.

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reciprocal control on the release of their neurotransmitters (Stoof et al., 1992a; DeBoer & Abercrombie, 1996; Pisani et al., 2003; Rice & Cragg, 2004; Cragg, 2006; Threlfell et al., 2012). A particularly important site of functional interaction in striatum is at autonomous pacemaker ChIs, the main source of striatal acetylcholine (ACh). ChIs express high levels of dopamine D2Rs and M2/M4 mACh autoreceptors (Yan & Surmeier, 1996; Straub et al., 2014; Wieland et al., 2014). D2Rs and M2/M4 mAChRs reduce tonic firing of ChIs, and subsequent ACh release, through shared coupling to $G_{i/o}$ signaling, which diminishes the opening of Cav2 Ca^{2+} channels in response to membrane depolarization (Yan & Surmeier, 1996; Yan et al., 1997; Calabresi et al., 1998; Maurice et al., 2004; Ding et al., 2006; Bonsi et al., 2008; Zhao et al., 2016). *In vivo* microdialysis (Drukarch et al., 1990; Stoof et al., 1992b; DeBoer & Abercrombie, 1996) and electrophysiological (Yan et al., 1997) studies revealed that the ability of the D2R agonist quinpirole to decrease ACh release and to reduce Ca^{2+} currents in ChIs is limited by increasing levels of striatal ACh, suggesting that dopamine modulation of striatal ACh release is influenced by striatal cholinergic tone, perhaps through functional interactions between D2R and M2/M4 mACh autoreceptors. Defining this interaction will have significant impact on understanding the most common basal ganglia motor disorders which are often associated with alterations in the normal balance between dopaminergic and cholinergic systems (Pisani et al., 2007; Aosaki et al., 2010; Bonsi et al., 2011; Benarroch, 2012). Dystonia, a common motor disorder characterized by abnormal muscle contractions, is of particular relevance in this context because of abundant evidence for alterations in striatal cholinergic-dopaminergic balance (Breakefield et al., 2008; Eskow Jaunarajs et al., 2015).

DYT1 dystonia, the most common early-onset inherited form of isolated dystonia, is caused by a three-nucleotide (GAG) deletion in the *TOR1A* gene, leading to loss of a glutamate residue near the C-terminus of the encoded protein torsinA, Δ*E*-TorA (Ozelius et al., 1999). TorA belongs to the AAA+ (ATPase associated with diverse cellular activities) family of chaperones and is involved in trafficking of proteins between the nucleus and endoplasmic reticulum. How these cell biological properties relate to neural system disturbances seen in dystonia are not well understood (Breakefield et al., 2008).

Mounting evidence suggests imbalanced cholinergic transmission plays a pivotal role in the pathophysiology of DYT1 dystonia (Eskow Jaunarajs et al., 2015). Clinically, anticholinergic drugs (*e.g.*, trihexyphenidyl) are the most effective treatments available for DYT1 and other dystonias (Jankovic, 2013). Across several rodent models based on mutant TorA, increased cholinergic signaling underlies impaired bidirectional corticostriatal synaptic plasticity, and comparable abnormalities in neural plasticity of motor circuits are found in human dystonia (Martella et al., 2009; Quartarone & Hallett, 2013; Martella et al., 2014; Calabresi et al., 2016). A consistent finding in all DYT1 rodent models examined so far is a "paradoxical excitation" of ChIs in response to D2R activation (Pisani et al., 2006; Martella et al., 2009; Sciamanna et al., 2012). This finding has led to the prevailing hypothesis that faulty D2R signaling causes the hypercholinergic state, with downstream effects on synaptic plasticity at corticostriatal synapses (Martella et al., 2009; Maltese et al., 2014; Martella et al., 2014; Calabresi et al., 2016). These clinical and experimental data predict that there should be elevated striatal cholinergic tone in DYT1 dystonia, but to date, ACh levels have not been measured directly *in vivo* in preclinical dystonia models or human dystonia.

Here, we provide direct evidence of elevated striatal cholinergic tone in a DYT1 knock-in mouse model ($TorA^{\Delta E/+}$ mice) and challenge the view that D2R signaling causes the hypercholinergic state. In fact, the reverse is true: we find that the strength of the cholinergic tone dictates the polarity of D2R modulation of ChI excitability. Moreover, our data support the interpretation that, as a result of the hypercholinergic state, a sustained activation of mACh autoreceptors on ChIs drives a shift from canonical D2R-$G_{i/o}$ coupling, which mediates the inhibition of ChI firing (Yan et al., 1997), to non-canonical D2R-β-arrestin signaling which triggers the aberrant increase in ChI firing. These findings not only provide a new perspective on DYT1 dystonia pathophysiology, but also have relevance to broader aspects of G-protein-coupled receptor signaling in conditions of dopamine/ACh imbalance found in a wide range of neurological disorders, including Parkinson's disease, Huntington's disease, l-DOPA induced dyskinesia and schizophrenia.

2. Materials and methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

2.1. Animal model

Heterozygous DYT1 mutant knock-in mice, $TorA^{\Delta E/+}$ (Goodchild et al., 2005) were maintained congenically by breeding with C57BL/6 J mice from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed with a 12 h light/dark cycle. Food and water were provided *ad libitum*. Tail DNA was genotyped using a primer pair to detect the 34 base pair loxP site in the DYT1 mutant (forward primer, AGTCTGTGG CTGGCTCTCCC; reverse primer, CCTCAGGCTGCTCACAACCAC). PCR products were run using a 2% agarose gel. Male mutant ($TorA^{\Delta E/+}$) and littermate controls ($TorA^{+/+}$) were evaluated between 4 and 6 months of age. The generation of β-Arr2 homozygous KO mice has been previously described (Bohn et al., 1999). These mice have been backcrossed >12 generations to and maintained on the C57BL/6 background.

2.2. In vivo microdialysis

Male $TorA^{+/+}$ and $TorA^{\Delta E/+}$ littermates were anesthetized with isoflurane (1–3%) and placed in a stereotaxic apparatus. Unilateral microdialysis cannulae (CMA Microdialysis, Stockholm, Sweden) were implanted vertically above the striatum (anterior +0.6; lateral +1.9; ventral −1.6 mm from bregma, according to the coordinates of (Paxinos et al., 1985). Two anchor screws (CMA Microdialysis) were placed behind the cannula and then fixed to the skull with dental cement. Following surgery, buprenorphine (0.03 mg/kg, i.p.) was injected for pain relief. Three to four days following surgery, dummy cannulae were removed and microdialysis probes were inserted (CMA7: 2 mm, CMA Microdialysis). Mice were habituated to the microdialysis environment for 3 h while the probes were perfused with artificial cerebrospinal fluid (aCSF; 127.6 mM NaCl, 4.02 mM KCl, 750uM NaH2PO4, 2.1 mM Na2HPO4, 2.00 mM MgCl2, 1.71 mM CaCl2; pH 7.4) at a constant rate of 2 μL/min for the duration of the experiment. In order to determine basal levels of ACh, three dialysate samples were collected every 20 min for one h following habituation. Following baseline, 10, 100 and 500 nM neostigmine bromide (Neo) were added to aCSF and samples were collected every 20 min for 1 h per dose in an escalating dose design for a total of 3 h. At the end of sampling, mice were killed and brains were removed and post-fixed with 4% paraformaldehyde. Fixed brains were then sectioned using a freezing microtome (Leica SM 200R, Buffalo Grove, IL) and cresyl violet staining was used to determine accurate probe placement. All microdialysis samples were analyzed for ACh using HPLC-ED (Eicom HTEC-500 with Eicom Autosampler INSIGHT, San Diego, CA) with an enzyme reactor (Eicom, AC-ENZYM II 1.0 × 4.0 mm) and a platinum electrode (Eicom, WE-PT; +450 mV *vs* Ag/AgCl). The mobile phase consisted of 50 mM potassium bicarbonate, 134 μM ethylenediaminetetraacetic acid, and 1.22 mM decanesulfonate (pH 8.5) and was delivered at a rate of 150 μL/min. Microdialysate sample chromatograms were analyzed based on established concentration curves for ACh (1–100 nM). The limit of detection was ~10 fM.