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Pharmacological evidence of functional inhibitory metabotrophic glutamate receptors on mouse arousal-related cholinergic laterodorsal tegmental neurons

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ABSTRACT

Cholinergic neurons of the pontine laterodorsal tegmentum (LDT) are importantly involved in neurobiological mechanisms governing states of arousal such as sleep and wakefulness as well as other appetitive behaviors, such as drug-seeking. Accordingly, mechanisms controlling their excitability are important to elucidate if we are to understand how these LDT neurons generate arousal states. Glutamate mediates the vast majority of excitatory synaptic transmission in the vertebrate CNS and while presence of glutamate input in the LDT has been shown and ionotropic responses to glutamate have been reported in the LDT, characterization of metabotropic responses is lacking. Therefore, electrophysiological responses and changes in levels of intracellular Ca^{2+} in mouse cholinergic LDT neurons following application of specific mGluR agonists and antagonists were examined. Unexpectedly, both the $mGluR_5$-specific agonist, CHPG, and the group II mGluR ($mGlu_{2/3}$) agonist, LY379268 (LY), induced a TTX-insensitive outward current/hyperpolarization. Both outward currents were significantly reduced by the mGluR antagonist MCPG and the CHPG-induced current was blocked by the specific $mGluR_5$ antagonist MTEP. Concurrent Ca^{2+}imaging revealed that while CHPG actions did include release of Ca^{2+} from CPA/thapsigargin-sensitive intracellular stores, actions of LY did not. Both CHPG- and LY-induced outward currents were mediated by a TEA-sensitive potassium conductance. The large-conductance, Ca^{2+}-dependent potassium (BK) channel blocker, iberiotoxin, attenuated CHPG actions. Consistent with actions on the BK conductance, CHPG enhanced the amplitude of the fast component of the after hyperpolarizing potential, concurrent with a reduction in the firing rate. We conclude that stimulation of $mGluR_5$ and group II ($mGluR_{2/3}$) elicits postsynaptically-mediated outward currents/hyperpolarizations in cholinergic LDT neurons. Effects of glutamatergic input would be, thus, expected not only to be excitation via stimulation of ionotropic glutamate receptors and $mGluR_1$, but also inhibition via actions at $mGluR_5$ and $mGluR_{2/3}$ on these neurons. As these two processes counteract each other, these surprising findings necessitate revision of predictions regarding the net level of excitation generated by glutamate input to cholinergic LDT cells and, by extension, the functional outcome of glutamate transmission on processes which these neurons regulate.

This article is part of a Special Issue entitled 'Metabotropic Glutamate Receptors'.

1. Introduction

States of arousal are regulated by concerted neuronal activity across many different brain regions; however, cholinergic neurons of the pontine laterodorsal tegmentum (LDT) are believed to be pivotal in behavioral state control via their extensive afferent output directed to both rostral and caudal CNS targets. Comprising an integral component of the reticular activating system (for review please see Steriade and McCarley (2005)), these pontine cells are directly involved in controlling naturally-occurring bouts of sleep and wakefulness (for review please see Lydic and Baghdoyan (2005)). Moreover, they control several anesthetized states, as the actions of many anesthetics include decreases in cholinergic transmission within the region of the pons wherein the LDT is located (Hambrecht-Wiedbusch et al., 2010; Keifer et al., 1996; Lydic and Baghdoyan, 2002; Mortazavi et al., 1999; Naruo et al., 2005). LDT neurons play a key role in motivated, goal-directed behaviors, such as drug seeking during highly-aroused states (Grace et al., 2007). In this context, an intact LDT is essential for behaviorally-relevant firing patterns of ventral tegmental (VTA) dopamine neurons which impart stimulus saliency (Lodge and Grace, 2006). Endogenous acetylcholine (ACh) dramatically modifies VTA dopamine neuronal firing patterns *in vivo*

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(Mameli-Engvall et al., 2006) and a prominent cholinergic input is provided by the LDT to preferentially dopamine-containing VTA neurons (Omelchenko and Sesack, 2005, 2006; Semba and Fibiger, 1992). Since activity of cholinergic LDT neurons plays such an important role in arousal regulation, if we are to understand the neurobiology underlying behavioral state control, determination of factors that control the cellular excitability of these cells, thus profoundly influencing cellular output to target regions, is of critical import.

Glutamate mediates the vast majority of excitatory synaptic transmission in the vertebrate CNS. The LDT receives excitatory, glutamatergic input deriving from outside the nucleus (Semba and Fibiger 1992; Sesack et al., 1989) and anatomical studies provide evidence for glutamatergic afferents directed to cholinergic neurons within the LDT (Clements et al., 1991; Honda and Semba, 1995; Semba and Fibiger, 1992). The LDT is a heterogeneous nucleus comprised not only of cholinergic cells but also separate populations of glutamate and GABA-containing cells (Wang and Morales, 2009), and we recently provided functional evidence that local LDT cells send glutamatergic input to neighboring cholinergic neurons (Kohlmeier et al., 2007). Presence of intra- and extra-nuclear sourced glutamate input directed to cholinergic neurons within the LDT begs further characterization of the response of cholinergic neurons to glutamate transmission.

Actions of glutamate are effectuated through two classes of receptors: the ionotropic and G-protein-coupled metabotropic receptors (mGluRs) (Conn and Pin, 1997). Ionotropic glutamate receptors have been well established as functionally involved in control of excitability of cholinergic LDT neurons. Immunocytochemical examinations have demonstrated presence of AMPA, kainate and NMDA receptor subunits co-localized with NADPH diaphorase, a marker of cholinergic LDT cells (Inglis and Semba, 1996). Moreover, electrophysiological studies have demonstrated that ionotropic receptors are present in the LDT (Imon et al., 1996; Sanchez et al., 1998; Stevens et al., 1992) and can be post-synaptically activated on cholinergic neurons (Sanchez et al., 1998). Activation of NMDA and AMPA receptors on cholinergic LDT neurons results in strong depolarization sufficient to drive firing (Sanchez et al., 1998) and AMPA agonists increase intracellular calcium levels – probably secondarily by activation of voltage-gated calcium channels arising from cell depolarization (Kohlmeier and Leonard, 2006) – consistent with the findings that postsynaptic actions of ionotropic glutamate receptors on cholinergic LDT neurons are always excitatory.

However, the presence, and functional cellular consequences of activation, of mGluRs on cholinergic LDT neurons have not yet been a focus of a published study. Currently, there are 8 known mGluR subtypes (1–8) which have been classed into 3 groups, Group I ($mGluR_{1\ \&\ 5}$), Group II ($mGluR_{2\ \&\ 3}$) and Group III ($mGluR_{4,6,7\ \&\ 8}$), based on similarities in amino acid sequence, pharmacology and second messenger system activation. Although the presence of mGluRs on cholinergic or noncholinergic neurons within the LDT has not been presented in a peer-reviewed format, the Allen Brain Atlas has provided evidence for gene expression of $mGluRs_{1,2,3,4,5\ \&\ 8}$ in the murine LDT region (www.brain-map.org). Cellular actions resulting subsequent to stimulation of mGluRs in the LDT are hard to predict and require experimental delineation, as activation of these receptors in other cells types results in a multitude of effects including modulation of second messenger levels, ion-channel activity, and synaptic efficacy (Conn and Pin, 1997; Nakanishi, 1994; Pin and Duvoisin, 1995). Although exceptions have been noted (Fagni et al., 1991; Fiorillo and Williams, 1998; Harata et al., 1996; Katayama et al., 2003), in the majority of cases, membrane potential effects of mGluR stimulation are depolarizing, either through inhibition of potassium conductances (Charpak and Gahwiler, 1991; Charpak et al., 1990) or activation of non-selective cation conductances (Glaum and Miller, 1992; Staub et al., 1992). Consistent herewith, preliminary studies indicated that in some cholinergic LDT cells, activation of mGluRs with the mGlu group I/II agonist, t-ACPD, elicits depolarization (Kohlmeier et al., 2008).

Because cholinergic transmission from LDT neurons modulates arousal levels and these cells receive significant glutamatergic input, it is of interest to extend our knowledge of the electrophysiological actions resulting from stimulation of glutamate receptors on cholinergic LDT neurons. To this end, we conducted whole-cell voltage and current-clamp recordings of identified cholinergic neurons from mouse brain slices to investigate the electrophysiological effects of stimulation of group I and II mGluRs, using subtype-specific agonists and antagonists. While in confirmation of earlier findings that application of a $mGluR_1$ agonist elicits membrane depolarization (Kohlmeier et al., 2008), contrary to our expectations, application of $mGluR_5$ and $mGluR_{2/3}$ agonists had inhibitory actions on cholinergic LDT cells. We conclude that this inhibitory cellular action of mGluR stimulation, in combination with the known excitatory actions of glutamate at $mGluR_1$ and ionotropic receptors could play a large role in the cellular mechanisms that govern behaviors mediated by glutamatergically-driven activity of cholinergic LDT neurons such as sleep, wakefulness, goal-directed and drug-seeking states, and may represent a pharmacological target for treatment of disorders associated with an inappropriate state of arousal.

2. Material and methods

2.1. Animals

All animal studies complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with Danish laws regulating experiments on animals. Animal use studies were permitted by the Animal Welfare Committee, appointed by the Danish Ministry of Justice, after determination that efforts had been extended to reduce the total numbers of animals used, to explore alternatives to use of animal tissue and to minimize animal suffering. The methods for preparing mouse brain slices, whole-cell recording, immunocytochemistry and high-speed calcium imaging, optimized for recording from cholinergic LDT neurons, are modified from those utilized previously (Burlet et al., 2002; Kohlmeier and Leonard, 2006; Kohlmeier et al., 2008) and are briefly summarized here.

Brain slices for whole-cell recordings and *bis*-Fura 2 calcium imaging (defined below) were obtained from 14 to 32 day old NMRI mice (Taconic, Denmark). Brain slices (250 μm) were prepared in ice-cold artificial cerebrospinal fluid (ACSF) that contained (in mM): 121 NaCl, 5 KCl, 1.2 NaH_2PO_4, 2.7 $CaCl_2$, 1.2 $MgSO_4$, 26 $NaHCO_3$, and 20 dextrose, which was oxygenated by bubbling with carbogen (95% O_2 and 5% CO_2). Slices containing the LDT were incubated at 35 °C for 15 min in oxygenated ACSF, and were then allowed to equilibrate to room temperature for 1 h. Following this period, the slice was transferred to a recording chamber and immersed in 21 °C ACSF, which was circulated through the chamber at a flow rate of 4–6 ml/min. In those cases where low calcium ACSF was utilized, calcium was buffered by inclusion of EGTA to approximately 20 μM as calculated using Patcher's Powers tools (Frank Würriehausen, MPI Biophysical Chemistry, Göttingen, Germany).

2.2. Whole-cell patch-clamp recordings

Electrodes for whole-cell patch-clamp recordings were prepared from thin-walled, borosilicate glass (6–11 MΩ, item number TW150F-4, WPI) with a Sutter P-97 horizontal puller (Sutter Instruments, USA). The electrode was filled with an internal solution of: (in mM) 144 K-Gluconate, 0.2 EGTA, 3 $MgCl_2$, 10 HEPES, 0.3 NaGTP, 4 Na_2ATP. When calcium imaging was being conducted, the potassium salt of Fura-2 (*bis*-Fura 2, 50 μM, Molecular Probes) was substituted for EGTA. In all cases, ATP was added right before use of the patch solution and biotinylated Alexa-594 (25 μM, Molecular Probes, Invitrogen) was also included in the solution so cells could be histochemically identified post hoc. Whole-cell patch-clamp recordings were obtained with an EPC-9 patch-clamp amplifier computer-controlled by Pulse version 8.8 (HEKA, Lambrecht/Pfalz, Germany) and regions for recording were selected from within the boundary of the LDT using a 4× objective on an Olympus BX50WI (USA) microscope with brightfield illumination. Neurons were then visualized using a Sensicam VGA camera (PCO imaging, Germany) under DIC optics with a 40× water immersion objective (NA 0.8, Olympus, USA). Although size is not an absolute indicator of acetylcholine (ACh) presence in LDT neurons, cholinergic LDT neurons tend to be larger than the GABA-containing cells in this nucleus