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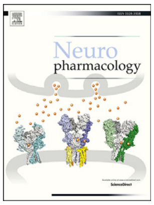

Calpain inhibition reduces NMDA receptor rundown in rat substantia nigra dopamine neurons

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ABSTRACT

Repeated activation of *N*-Methyl-D-aspartate receptors (NMDARs) causes a Ca^{2+}-dependent reduction in NMDAR-mediated current in dopamine (DA) neurons of the substantia nigra pars compacta (SNc) in one week old rats; however, a Ca^{2+}-dependent regulatory protein has not been identified. The role of the Ca^{2+}-dependent cysteine protease, calpain, in mediating NMDAR current rundown was investigated. In brain slices from rats aged postnatal day 7–9 (‘P7’), bath application of either of the membrane permeable calpain inhibitors, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN, 20 μM) or MDL-28170 (30 μM) significantly reduced whole-cell NMDAR current rundown. To investigate the role of the calpain-2 isoform, the membrane permeable calpain-2 inhibitor, Z-Leu-Abu-CONH-CH2-C6H3 (3, 5-(OMe)2 (C2I, 200 nM), was applied; C2I application significantly reduced whole cell NMDAR current rundown. Interestingly, ALLN but not C2I significantly reduced rundown of NMDA-EPSCs. These results suggest the calpain-2 isoform mediates Ca^{2+}-dependent regulation of extrasynaptic NMDAR current in the first postnatal week, while calpain-1 might mediate rundown of synaptic NMDAR currents. One week later in postnatal development, at P12-P16 (‘P14’), there was significantly less rundown in SNc-DA neurons, and no significant effect on rundown of either Ca^{2+} chelation or treatment with the calpain inhibitor, ALLN, suggesting that the rundown observed in SNc-DA neurons from two week-old rats might be Ca^{2+}-independent. In conclusion, Ca^{2+}-dependent rundown of extrasynaptic NMDAR currents in SNc DA neurons involves calpain-2 activation, but Ca^{2+}- and calpain-2-dependent NMDAR current rundown is developmentally regulated.

1. Introduction

N-Methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that play important roles in neuronal development and plasticity (Cull-Candy and Leszkiewicz, 2004; Luscher and Malenka, 2012; Paoletti et al., 2013; Traynelis et al., 2010; Wyllie et al., 2013). One important functional feature of NMDARs is their Ca^{2+} permeability, which results in activation of intracellular signalling pathways, including those that promote cell survival (Hardingham and Bading, 2010; Traynelis et al., 2010; Vyklicky et al., 2014). However, over-activity of NMDARs and the resulting excessive Ca^{2+} influx are also implicated in neuronal dysfunction and excitotoxicity through the activation of cell death signalling pathways (Choi, 1987; Hardingham and Bading, 2010; Surmeier et al., 2010). This mechanism could be a contributing factor to neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's diseases (Parsons and Raymond, 2014; Ambrosi et al., 2014).

NMDARs are themselves regulated by Ca^{2+}-dependent processes, including a Ca^{2+}-dependent reduction of NMDAR current, termed inactivation or rundown (Ehlers et al., 1996; Legendre et al., 1993; Rosenmund et al., 1995). NMDAR current rundown has previously been shown in substantia nigra pars compacta (SNc) dopaminergic (DA) neurons when NMDA was repetitively applied at an interval less than 400 s (Wild et al., 2014). Rundown was dependent on Ca^{2+} influx as it was reduced by increasing the concentration of intracellular buffer (from 0.6 mM EGTA to 10 mM BAPTA), by replacing extracellular Ca^{2+} ions with Ba^{2+} ions, or by reducing the driving force for Ca^{2+} entry by depolarising the membrane potential to +40 mV (Wild et al., 2014). The Ca^{2+}-dependent regulatory mechanism has not been identified. SNc DA neurons form a critical part of the basal ganglia circuitry involved in

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voluntary motor control (Hegarty et al., 2013), and their spiking is strongly influenced by glutamatergic inputs and NMDAR activity (Blythe et al., 2007; Jones et al., 2011). Degeneration of SNc-DA neurons is a pathological hallmark of Parkinson's disease (Obeso et al., 2010; Beitz, 2014), and deregulated Ca^{2+} influx is a possible contributing factor to DA neuronal death (Surmeier et al., 2010, 2011). Therefore, there is considerable interest in identifying the factors that regulate NMDAR activity in these and other brain neurons.

Calpain is a Ca^{2+}-dependent cysteine protease, which is activated upon NMDAR stimulation, and calpain is widely expressed in the mammalian central nervous system (Baudry and Bi, 2016). Calpain has been shown to regulate NMDAR activity in synaptic membranes (Bi et al., 1998) and over-activation of NMDARs results in a calpain-dependent suppression of NMDAR currents (Wu et al., 2005). Calpain is also implicated in the pathophysiology of several neurological diseases such as traumatic brain injury and ischemia (Goll et al., 2003; Curcio et al., 2016). While 15 members have been identified in the calpain family (Sorimachi et al., 2010), the two major isoforms expressed in the brain are calpain-1 (μ-calpain) and calpain-2 (m-calpain) (Doshi and Lynch, 2009; Baudry and Bi, 2016). These two isoforms exhibit differing Ca^{2+} sensitivity (micromolar versus milimolar concentrations of Ca^{2+} respectively; Dayton, 1982), and recent studies have suggested that they play opposite functions in synaptic plasticity and neuronal death (Baudry and Bi, 2016). Calpain expression is also isoform-dependent, with calpain-1 being expressed in neuronal somata and calpain-2 expressed in axonal processes and glial cells (Siman et al., 1985). Furthermore, higher levels of calpain are found in hindbrain and midbrain structures compared with forebrain structures in rats (Simonson et al., 1985). Calpain is present in SNc-DA neurons in the adult rat (Siman et al., 1985), although in human post-mortem brains, calpain-2 was detected only in the substantia nigra of Parkinsonian (PD) patients, not in control subjects (Mouatt-Prigent et al., 1996), raising the possibility that calpain-2 might be upregulated in PD. In this study, the role of calpain in Ca^{2+}-dependent NMDAR current rundown in SNc-DA neurons was investigated using two inhibitors that target both of these calpain isoforms (ALLN and MDL-28170) and one inhibitor more selective for the calpain-2 isoform, Z-Leu-Abu-CONH-CH2-C6H3 (3, 5-(OMe) 2 (C2I) (Wang et al., 2014), and a significant reduction in NMDAR current rundown was observed.

2. Materials and methods

Wistar rats (Charles River) aged postnatal day 7–16 were used in all experiments in accordance with the Animals (Scientific procedures) Act 1986 and with the University of Cambridge Animal Welfare and Ethical Review Board. Animals were housed in 12 h light/dark cycle with up to 8 pups per dam and *ad libitum* access to food and water. Rats were anesthetized with isofluorane, then decapitated and brains were removed and submerged in ice-cold solution containing (mM): NaCl 52.5, sucrose 100, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 25, $MgCl_2$ 5, $CaCl_2$ 1, kynurenic acid 0.1; pH 7.4 with 95% O_2/5% CO_2. Horizontal slices (250–300 μm) containing the substantia nigra were made using a Campden 7000smz Vibrating Microtome (Campden Instruments, UK). Slices were then transferred to an incubation chamber containing (mM): NaCl 119, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, glucose 25, $MgCl_2$ 5, $CaCl_2$ 2 at 30 °C, saturated with 95% O_2 and 5% CO_2, and left undisturbed for 45 min to 5 h.

Brain slices were transferred to a recording chamber and perfused at 2–3 mL/min with oxygenated solution (as stated above except with 10 mM glucose and 0.1 mM $MgCl_2$) at 30 ± 2 °C. In some experiments, no $CaCl_2$ was added to the recording solution and instead 2 mM $BaCl_2$ was added (nominally Ca^{2+}-free solution). Patch pipettes with a tip resistance of 1.5–3.5 MΩ were filled with intracellular solution containing (mM): $CsMeSO_3$ 120, CsCl 5, NaCl 2.8, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20, $MgCl_2$ 3, $CaCl_2$ 0.5, Mg-ATP (adenosine triphosphate) 2, Na-GTP (guanine triphosphate) 0.3, Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) 0.6, EGTA 10 or 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA) 10, as indicated in the text (pH 7.2–3, 270–290 mOsm). Series resistance was measured between each agonist application and was typically 3–5 MΩ; recordings were discarded if the series resistance was unstable. SNc-DA neurons were identified by visually locating the medial terminal nucleus of the accessory optic tract. DA neurons make up ~90% of the SNc neuronal population and exhibit a time-dependent, hyperpolarization-activated inward current (I_h) (of more than 50 pA) following a voltage step from −60 to −120 mV (Margolis et al., 2006; Wild et al., 2014; Neuhoff et al., 2002; Washio et al., 1999).

SNc-DA neurons were voltage clamped to −60 mV, −50 mV or +40 mV (as indicated in the text) using an Axopatch 200B patch clamp amplifier (Molecular Devices, USA). Whole-cell currents were elicited by agonist applications (NMDA 0.5 mM; 5 s in duration; 100 s interval; 10 psi) via a Picospritzer II (General Valve corporation), positioned ~200 μm away from the cell, in the presence of picrotoxin 50 μM, glycine 10 μM and tetrodotoxin (TTX) 100 nM. Application via the picospritzer results in ~50% dilution to ~250 μM NMDA (Wild et al., 2013). Synaptic NMDARs were stimulated using a bipolar stainless steel electrode (Frederick Haer and Co., USA); stimuli (100 μs duration, amplitude 60–250 μA) were applied every 10 s in the presence of picrotoxin (50 μM), glycine (10 μM) and DNQX (10 μM). Drugs were applied to the perfusion solution immediately after the visualization of the I_h; the first NMDA application occurred at least 300 s after drug application.

Data were recorded and analysed using Spike 2 software (Version 4; Cambridge Electronic Design, Cambridge, UK). To quantify the extent of rundown, peak current amplitude in response to electrical stimulation or agonist application was measured. The ratio of the average NMDAR-EPSC recorded at 500–600 s to the average NMDAR-EPSC at 0–100 s ($I_{t500-600s}$/$I_{t(0-100s)}$), or the ratio of the last agonist-induced response to the first response (I_{t300}/I_{t0}) was calculated. Statistical analyses were carried out using GraphPad Prism (version 4.0, La Jolla California, USA). Data distribution was assessed using the Shapiro Wilk Normality test. When comparing two groups of paired or unpaired data, the paired or unpaired Student's t-test or a non-parametric test were used. For more than two groups of data, one-way ANOVA with Tukey's post-tests were used. Data are expressed as mean ± standard error (SE), and ‘n’ values indicate the number of cells, which is equivalent to the number of slices.

3. Results

3.1. Repeated agonist application leads to Ca^{2+}-dependent NMDAR current rundown

NMDAR current rundown was confirmed in SNc-DA neurons from P7-P9 (‘P7’) rats by four consecutive agonist applications. Neurons were voltage clamped to −60 mV with the Ca^{2+} chelator EGTA (0.6 mM) in the intracellular pipette solution. Under these conditions, the peak inward current decreased in amplitude after the first agonist application (Fig. 1A, B, E). The initial current response to NMDA application (I_{t0}) had a mean amplitude of 1657 ± 278 pA, while the fourth response (I_{t300}) was significantly reduced, with a mean amplitude of 785 ± 110 pA (n = 16 cells from 15 rats; $P = 0.0005$; Wilcoxon signed rank test; Fig. 1B). Rundown