Persistent elevation of D-Aspartate enhances NMDA receptor-mediated responses in mouse *substantia nigra pars compacta* dopamine neurons

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ABSTRACT

Dopamine neurons in the *substantia nigra pars compacta* regulate not only motor but also cognitive functions. NMDA receptors play a crucial role in modulating the activity of these cells. Considering that the amino-acid D-Aspartate has been recently shown to be an endogenous NMDA receptor agonist, the aim of the present study was to examine the effects of D-Aspartate on the functional properties of nigral dopamine neurons. We compared the electrophysiological actions of D-Aspartate in control and D-aspartate oxidase gene ($Ddo^{-/-}$) knock-out mice that show a concomitant increase in brain D-Aspartate levels, improved synaptic plasticity and cognition. Finally, we analyzed the effects of L-Aspartate, a known dopamine neuron endogenous agonist in control and $Ddo^{-/-}$ mice. We show that D- and L-Aspartate excite dopamine neurons by activating NMDA, AMPA and metabotropic glutamate receptors. *Ddo* deletion did not alter the intrinsic properties or dopamine sensitivity of dopamine neurons. However, NMDA-induced currents were enhanced and membrane levels of the NMDA receptor GluN1 and GluN2A subunits were increased. Inhibition of excitatory amino-acid transporters caused a marked potentiation of D-Aspartate, but not L-Aspartate currents, in $Ddo^{-/-}$ neurons. This is the first study to show the actions of D-Aspartate on midbrain dopamine neurons, activating not only NMDA but also non-NMDA receptors. Our data suggest that dopamine neurons, under conditions of high D-Aspartate levels, build a protective uptake mechanism to compensate for increased NMDA receptor numbers and cell hyper-excitation, which could prevent the consequent hyper-dopaminergia in target zones that can lead to neuronal degeneration, motor and cognitive alterations.

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1. Introduction

D-Aspartate (D-Asp), like its L-enantiomer (L-Aspartate; L-Asp) is an endogenous NMDA receptor ligand, binding to the glutamate site of the GluN1 and GluN2 subunits (Errico et al., 2012; Kiskin et al., 1990; Ota et al., 2012; Verdoorn and Dingledine, 1988). In addition to NMDA receptors, D-Asp can also activate mGlu$_5$ metabotropic glutamate receptors (Molinaro et al., 2010). D-Asp is present in the brain at high concentrations during embryogenesis and early life, accumulating in the soma, synaptic vesicles and

synaptosomes of neurons (Dunlop et al., 1986; Neidle and Dunlop, 1990; Wolosker et al., 2000). *In vitro* studies indicate that it can be released in a Ca^{2+}-dependent manner after electrical or chemical stimulation (Muzzolini et al., 1997; Palmer and Reiter, 1994; Savage et al., 2001) and its uptake is mediated by Na^+-dependent excitatory amino-acid transporters (EAATs; Adachi et al., 2004; Koyama et al., 2005).

D-Asp is produced by a not-yet identified mammalian aspartate racemase (Tanaka-Hayashi et al., 2015; Kim et al., 2010) and is catabolised by D-Aspartate oxidase (DDO; Katane and Homma, 2010). In fact, the distributions of D-Asp and DDO in the brain are opposed, with high levels of one correlating with low levels for the other (Schell et al., 1997). DDO is highly expressed in the adult brain resulting in low levels of endogenous D-Asp during adulthood (Katane and Homma, 2010). Thus, a targeted deletion of the *Ddo* gene in adult mice ($Ddo^{-/-}$) determines a 10–20-fold increase of D-Asp in different brain areas such as the cortex, striatum, hippocampus, cerebellum and olfactory bulb (Errico et al., 2006, 2008a,b; 2011a,b; Huang et al., 2006). As a direct consequence of D-Asp increase, $Ddo^{-/-}$ brains display high contents of endogenous NMDA, the N-methyl derivative of D-Asp (D'Aniello et al., 2000a,b; Errico et al., 2006).

Information from $Ddo^{-/-}$ mice was crucial for understanding the physiological role of D-Asp in the brain. For example, in CA1 hippocampal pyramidal cells, young adult $Ddo^{-/-}$ mice show enhanced NMDA receptor-dependent LTP and have enhanced cognitive properties, improved memory and spatial learning. In the hippocampus and the prefrontal cortex this is accompanied by increases in spine density and dendritic length (Errico et al., 2008a, 2011b, 2012, 2014). In accordance with an abnormally high NMDA receptor transmission, $Ddo^{-/-}$ mice are also characterized by loss of the corticostriatal long-term depression (Errico et al., 2008b, 2011a, 2012). It was also reported that in $Ddo^{-/-}$ mice, increased D-Asp contents since perinatal phases counteract phencyclidine-induced schizophrenia-like behaviours and cerebral dysfunction (Errico et al., 2013, 2015), pointing to a potential role for D-Asp in the therapeutics of schizophrenia.

Dopaminergic neurons (DAergic) in the *substantia nigra pars compacta* (SNc) are the main source of dopamine (DA) in the mammalian central nervous system. DA, released from these neurons, plays an important role in the control of multiple brain functions including voluntary movement and cognition (Matsumoto and Takada, 2013; Pasquereau and Turner, 2015) and alterations in the DAergic transmission have long been associated with neurological disorders such as Parkinson's disease and schizophrenia (Howes and Kapur, 2009; Przedborski, 2005). Unlike the better-characterised role of D-Asp in the striatum and the hippocampus, target areas of DAergic neurons (Fallon, 1981; Prensa and Parent, 2001; Scatton et al., 1980), there is scarce information on the role of D-Asp in the SNc. Thus, in the present study we investigated the role of D-Asp on the functional properties of SNc DAergic neurons. We used $Ddo^{-/-}$ mice to examine whether enhanced endogenous D-Asp levels in these animals can modify glutamate receptor expression and function, compared to control littermates ($Ddo^{+/+}$). Finally, we investigated whether changes in the D-Asp content of the $Ddo^{-/-}$ brain can alter the responses of DA neurons mediated by L-Asp, an endogenous agonist in the SNc (Abarca et al., 1995).

Our results unveiled an unexpected role of D-Asp in mediating not only NMDA but also non-NMDA receptor glutamatergic transmission in the SNc. We also show that, in conditions of increased endogenous levels of D-Asp, in $Ddo^{-/-}$ mice, NMDA currents are enhanced, membrane levels of NMDA receptor subunits are increased and that DAergic neurons compensate for these alterations by an enhanced mechanism of EAAT-mediated D-Asp

transport. Our data provide new insights into the physiological role of D-Asp in mediating glutamatergic transmission in the midbrain and suggest that D-Asp, by regulating the activity of DAergic neurons, could modulate DA transmission and affect motor, as well as, cognitive functions.

2. Material and methods

2.1. Animals

Animal usage adhered to ARRIVE guidelines and complied with the ethical guidelines of the European Commission Directive (2010/63/EU) and the Italian Health Ministry (Art.31, D.Lgs 26/2014; project protocol: DM55/2014-PR) and was approved by the Animal Ethics Committee of the Fondazione Santa Lucia. $Ddo^{+/+}$ and $Ddo^{-/-}$ mice (C57BL/6 background, P17–P23 or adults) were housed in a temperature- and humidity-controlled environment (free access to food and water; 12 h dark/light cycle). All efforts were made to minimize animal suffering and reduce the total number of animals used. A total of 55 animals (males and females) were used.

2.2. Acute brain slice preparation

Horizontal midbrain slices (230–300 μm; Leica VT1200S vibratome) were obtained following isoflurane or chloral hydrate anaesthesia (400 mg.kg^{-1}, i.p.) and quick decapitation. Brains were removed and placed in chilled bubbled (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) containing (mM): NaCl 126, NaHCO$_3$ 24, glucose 10, KCl 2.5, CaCl$_2$ 2.4, NaH$_2$PO$_4$ 1.2, MgCl$_2$ 1.2 (pH 7.4; 290 mOsm.L^{-1}). Slices were hemisectioned and incubated in aCSF (32–33 °C) for at least 40 min prior to usage.

2.3. Electrophysiological recordings

Slices chosen for recordings were transferred to the recording chamber (0.6 mL) of an upright microscope (Axioskop 2-FS; Zeiss) and were continuously perfused with aCSF (32–33 °C). Whole-cell recordings were conducted from P17–P23 mice. SNc DAergic neurons, visualized using infrared differential interference contrast, were identified as tightly packed, medium-sized cells adjacent to the medial terminal nucleus (MT). Whole-cell currents (−60 mV, with a MultiClamp 700B) or spontaneous cell firing (in current-clamp, I = 0 mode) were filtered at 1–4 kHz using the amplifier's in-built low-pass filter, digitised with Digidata 1322A and computer-saved using Clampex 9 (all from Molecular Devices) at a sampling rate of 4-times the filter frequency. Electrodes (3–5 MΩ), pulled from thin-wall filamented glass (World Precision Instruments) were filled with a solution containing (mM): 135 K-gluconate, 10 KCl, 10 HEPES, 2 MgCl$_2$, 4 ATP-Mg^{2+}, 0.3 GTP-Na$^+$, 0.1 EGTA, 0.045 CaCl$_2$ (pH 7.3, 280–290 mOsm.L^{-1}). DAergic neurons were further identified by the expression of a prominent hyperpolarisation-activated inward current (I$_h$) in response to hyperpolarizing voltage steps (from −60 mV to −120 mV at −20 mV intervals, 1 s), slow spontaneous firing (0.5–4 Hz) and a transient outward current after a brief application of dopamine (30 μM, 25–60 s) in voltage-clamp or after hyperpolarisation induced by quinpirole (1 μM) in current-clamp. NMDA (bath-applied; 20 or 50 μM) currents were recorded with or without Mg^{2+} in the pipette and aCSF solution, in the presence of bath-applied glycine (50 μM), picrotoxin (100 μM), NBQX (10 μM) and lidocaine (200 μM). Recordings with access resistance higher than 20 MΩ were discarded. No liquid junction potential correction was applied.

Extracellular recordings (non-visual) from adult mice (older than 2 months) were performed by slowly moving the recording

electrode (filled with aCSF, 4–8 MΩ) in the area surrounding the MT until firing was detected. DAergic SNc neurons were identified based on the following criteria: (a) slow, regular firing, (b) long spike duration and (c) reversible inhibitory response to bath application of DA. Spikes (I = 0 mode) were recorded with high-pass (0.5 Hz) and low-pass filtering (1 kHz) at 20 kHz sampling rate.

2.4. Drugs

All drugs (Abcam, UK) used for electrophysiology, prepared from thawed concentrated stocks, were either bath-applied or pressure-ejected. For whole-cell current recordings the duration of bath-application was determined as sufficient when the current reached a steady-state plateau (at low agonist concentrations) or a sag after a peak (at higher concentrations). The solution flow-rate was checked regularly (3–4 mL.min^{-1}). To study the pharmacology underlying the D- and L-Asp currents we performed experiments using a fast application system: pressure-ejected D- and L-Asp (3 mM) were applied to the slice from a low-resistance pipette using a PV800 Pneumatic PicoPump (WPI) at pressures of 5–20 psi and for 1–4 s, every 3 min. By means of this technique, absolute current amplitude and kinetics may vary among neurons, depending on factors such as the distance of the recorded cell from the slice surface (where the agonist ejection occurs) or the route of aCSF flow which removes the agonist. However, this agonist application system elicits stable currents up to several minutes, being thus suitable for the pharmacological analysis (Cucchiaroni et al., 2010; Guatteo et al., 1999; Ledonne et al., 2015; Tozzi et al., 2001). Agonist-induced currents were recorded in control conditions or in the presence of MK-801 (30 μM), CNQX (30 μM) and CPCCOEt (100 μM), bath-applied for 9–12 min, or in DL-TBOA (100 μM, bath-applied for 6 min). During extracellular recordings, all drugs were bath-applied for 3–13 min to establish stable firing.

2.5. Data analysis

The instantaneous firing frequency from extracellular recordings is the reciprocal of the event-to-event time interval, obtained using the threshold method (Clampfit 9). The mean firing frequency for each drug treatment was calculated for a period of 2–5 min of continuous, stable firing activity.

Real time membrane resistance (R_m) and membrane capacitance (C_m) were routinely monitored with 5 mV steps (50 Hz) in voltage-clamp at −60 mV holding potential. Whole-cell currents induced by the application of amino-acids were analyzed for peak amplitude and area. The I_h current amplitude (at −120 mV) was taken as the difference between the steady-state (at the end of the 1 s pulse) and the current onset (at the start of the 1 s pulse). Data were analyzed with Clampfit and plotted with Origin 6 (OriginLab).

2.6. Biotinylation assay and immunoblotting

Surface biotinylation was performed using "Pierce® Cell Surface Protein Isolation Kit" (Thermo Scientific 89881) as described by Ledonne et al. (2015), with modifications. Briefly, midbrain slices were incubated in bubbled aCSF containing 0.5 mg.mL^{-1} EZ-Link® Sulfo–NHS–SS-Biotin for 45 min on ice. After incubation the SNc region was isolated and washed with ice-cold aCSF, followed by ice-cold TBS (25 mM Tris, 150 mM NaCl, pH 7.2). Samples were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, 300 mM sucrose, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail) with a pellet pestle and then sonicated/vortexed. After incubation on ice (15 min) the samples were centrifuged and the total protein content of the supernatant was measured by the Bradford method. For each sample, 200 μg of proteins diluted in

150 μL of lysis buffer were incubated with 150 μL NeutrAvidin® Agarose for 16 h at 4 °C on a rocking platform. After overnight incubation the columns were briefly centrifuged and washed with wash buffer. Biotinylated proteins were eluted by incubating the samples for 1 h in agitation (400 rpm at room temperature) with 100 μL sample buffer (50 mM Tris–HCl pH 6.8, 10% Glycerol, 1% SDS, 50 mM DTT) and then centrifuged.

Equal amount of total proteins (20 μg) and equal volume of biotinylated proteins (30 μL) were applied to SDS-PAGE and electroblotted on a polyvinylidene fluoride membrane. Immunoblotting analysis was performed using a chemiluminescence detection kit. The relative levels of immunoreactivity were determined by densitometry using ImageJ. Primary antibodies: GluN1 (1:1000; Santa Cruz sc-1467); GluN2A (1:1000, Santa Cruz sc-1468); GluN2B (1:1000, Millipore 06–600); GluA1, clone C3T (1:1000; Millipore 04-855); Actin (1:50000; Sigma–Aldrich A5060); Tyrosine hydroxylase (TH; 1:1000, Abcam ab112). Secondary antibodies: goat anti-rabbit IgG horseradish peroxidase-conjugate (1:3000; Bio-Rad 170-6515); rabbit anti-goat IgG horseradish peroxidase-conjugate (1:1000; Bio-Rad 172-1034).

2.7. Statistical analysis

Two-tailed paired or unpaired Student's t-tests, according to the data, were performed using PRISM 5.01 (GraphPad). Results are considered significant for $P \leq 0.05$. Data are expressed as average values ± sem.

3. Results

3.1. Functional characterization of intrinsic electrophysiological properties of $Ddo^{-/-}$ DAergic neurons

We first examined the intrinsic properties of SNc DAergic neurons in $Ddo^{+/+}$ and $Ddo^{-/-}$ midbrain slices to test if they are affected by increased levels of D-Asp in $Ddo^{-/-}$ mice. DAergic cells are spontaneously active in vitro, firing action potentials in a slow, regular pattern (Grace and Onn, 1989; Lacey et al., 1989). Accordingly, during extracellular recordings in adult mice (older than 2 months), we observed a characteristic pacemaking pattern both in $Ddo^{+/+}$ and $Ddo^{-/-}$ neurons (Fig. 1A). Although there was a small tendency for $Ddo^{-/-}$ cells to fire faster, the mean firing frequency was not significantly different from that of $Ddo^{+/+}$ cells. All cells were temporary silenced by brief bath application of DA (25–60 s), indicating that the Ddo deletion does not affect dopamine sensitivity.

Whole-cell recordings in $Ddo^{+/+}$ and $Ddo^{-/-}$ neurons (−60 mV; P17-23) revealed similar amplitudes of the I_h current (Fig. 1B) which is characteristically prominent in these cells (Mercuri et al., 1995). Mean values of membrane resistance (R_m) and cell capacitance (C_m) were also similar in the two genotypes (Fig. 1C). Thus, the basic behaviour of SNc neurons is unchanged in $Ddo^{-/-}$ mice compared to controls.

3.2. Effects of exogenous D-Asp on glutamatergic receptors in SNc DAergic neurons

Considering that $Ddo^{-/-}$ mice show increased brain levels of D-Asp (Errico et al., 2006; Huang et al., 2006), the results shown in Fig. 1 were surprising: the lack of an apparent effect of excessive D-Asp in $Ddo^{-/-}$ neurons could be explained either with the notion that this amino-acid has no action on DAergic cells or with the possibility that, in our acute preparations perfused with aCSF, the excessive D-Asp in $Ddo^{-/-}$ slices could be washed away and subtle transient effects, such as changes in firing, would not be evident.

A

+/+   -/-

DA 30 µM

0.5 mV

5 s

Mean Inst. FF (Hz)

(29)   (31)

+/+   -/-

B

60 mV

+/+

-/-

500 pA

500 ms

I_h current (pA)

+/+
-/-

(26) (26)

C

+/+
-/-

R_m (MΩ)

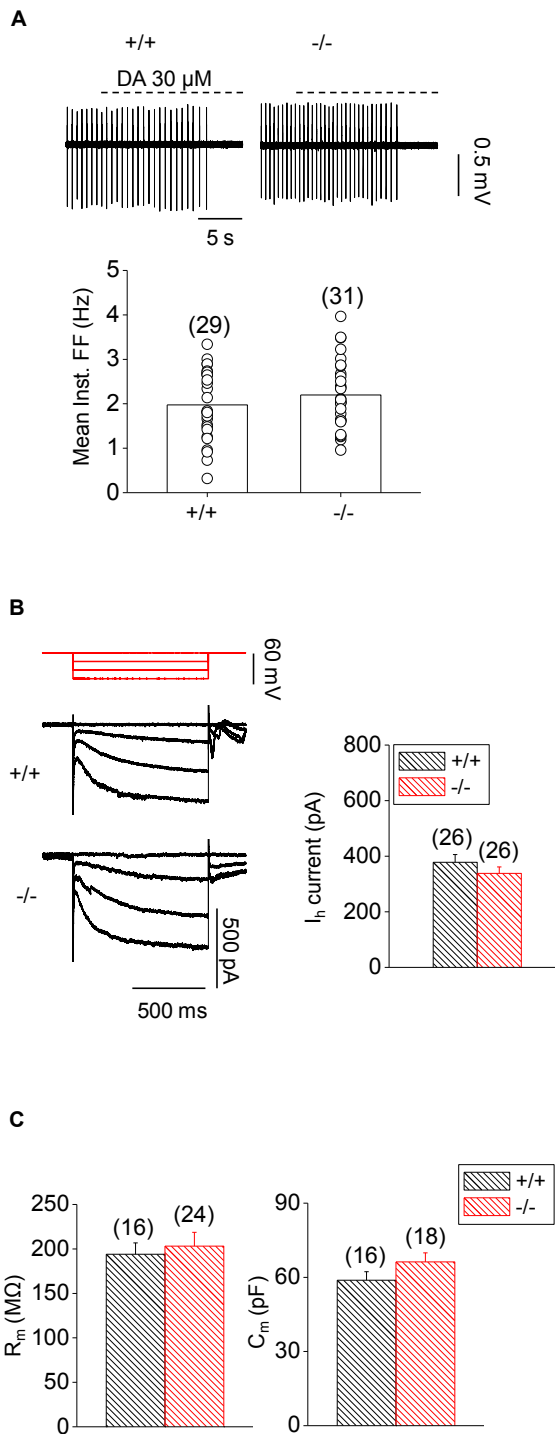(16) (24)

C_m (pF)

(16) (18)

Fig. 1. Genetic deletion of the *Ddo* gene does not affect intrinsic properties of nigral DA neurons. (A) Single-unit extracellular recordings showing spontaneous firing from $Ddo^{+/+}$ (+/+) and $Ddo^{-/-}$ (−/−) cells, temporarily silenced by 30 µM of dopamine (30 s bath application, top); the histogram shows the mean pulled instantaneous firing frequency ($P = 0.261$ with unpaired *t*-test), open circles show single-neuron data in the two cell types. (B) Example traces of hyperpolarisation-activated inward currents (I_h) from whole-cell recordings of DA neurons held at −60 mV, obtained with −20 mV hyperpolarisation steps (inset above traces). The pulled plot revealed that the I_h current amplitude, measured at −120 mV, is similar in the two genotypes ($P = 0.270$). (C) Mean values of membrane resistance (R_m) and cell capacitance (C_m) were comparable in knock-out and control DA neurons (R_m: $P = 0.671$; C_m: $P = 0.150$, unpaired *t*-test). Number of cells are in parenthesis.

We therefore performed experiments with exogenously applied D-Asp, firstly for proving its role as a glutamatergic agonist in DAergic neurons and, secondly, for unmasking potential long-lasting effects of the excessive D-Asp in $Ddo^{-/-}$ neurons, such as changes in glutamate receptor numbers, pharmacology and agonist affinity.

Bath application of D-Asp during whole-cell recordings (−60 mV) could indeed activate concentration-dependent inward currents in both $Ddo^{+/+}$ and $Ddo^{-/-}$ neurons (Fig. 2A), indicating that D-Asp can act as a potential endogenous agonist in DAergic neurons. As seen from the dose−response curves in Fig. 2A, there were no significant differences in current amplitude between $Ddo^{-/-}$ and $Ddo^{+/+}$ neurons at all concentrations tested, suggesting similar sensitivity for the agonist, regardless of genotype.

To study the pharmacology underlying the D-Asp currents we pressure-ejected D-Asp near a patched cell at a saturating concentration (Fig. 2B). Currents, induced every 3 min (to allow for full recovery from receptor desensitization) were recorded in control conditions and during the presence of glutamate antagonists. Our aim was to unmask the glutamate receptors types activated by D-Asp in $Ddo^{+/+}$ and $Ddo^{-/-}$ neurons and to calculate the contribution of each receptor type in the overall current. Examples of such experiments are shown in Fig. 2B, showing the D-Asp current being gradually reduced with the sequential addition of each antagonist. In the presence of MK-801 + CNQX + CPCCOEt almost no current was detected, indicating that D-Asp can activate both ionotropic and metabotropic glutamate receptors. As reported in the plot (Fig. 2B) the current produced by D-Asp is mainly NMDA receptor-dependent, since MK-801 caused the biggest reduction in the overall current. Although there was a tendency for MK-801 to be more potent in $Ddo^{-/-}$ cells, the difference from $Ddo^{+/+}$ neurons was not significant ($P = 0.178$; Fig. 2B). A smaller component of D-Asp-induced current was also mediated by AMPA/kainate and metabotropic $mGlu_{1/5}$ receptors, though, again, no differences were observed between the two genotypes ($P = 0.178$; $P = 0.480$, respectively).

Next, to study the effect of exogenous D-Asp on the spontaneous firing of DAergic neurons we used extracellular recordings that do not perturb the cytoplasmic content (Fig. 2C). The application of D-Asp caused a significant increase in firing that was reversed by AP5 in both genotypes. This is in agreement with the notion that exogenous D-Asp excites DAergic cells by mainly activating NMDA receptors. The plot in Fig. 2C summarizes results from all cells: the spontaneous firing frequency was significantly increased after D-Asp ($77 \pm 18\%$ increase in $Ddo^{+/+}$, $P = 0.002$; $143 \pm 65\%$ increase in $Ddo^{-/-}$, $P = 0.043$; paired Student's *t*-test) and then reduced to near-control levels by AP5 ($40 \pm 9\%$ decrease in firing frequency, $P = 0.042$ in $Ddo^{-/-}$ versus $25 \pm 7\%$, $P = 0.073$ in $Ddo^{+/+}$; paired Student's *t*-test). However, between the two genotypes, no statistical significance was reached for either treatment ($P = 0.394$ for D-Asp treatment; $P = 0.272$ for D-Asp + AP5; unpaired *t*-test).

To summarise, exogenous D-Asp acts as a glutamatergic agonist on DAergic neurons, by activating mainly NMDA but also non-NMDA receptors. Additionally, similar to our MK-801 data from whole-cell recordings, the results from extracellular recordings suggest that D-Asp mediates a stronger (though not significant) activation of NMDA receptors in $Ddo^{-/-}$ DA neurons compared to $Ddo^{+/+}$ cells.

3.3. Effects of L-Asp on glutamatergic receptors in SNc DAergic neurons

Since our experiments with D-Asp (Fig. 2) showed only small differences between $Ddo^{-/-}$ and $Ddo^{+/+}$ neurons, we decided to use a different glutamatergic agonist with known actions in the SNc. It has previously been reported that L-Asp is released in the
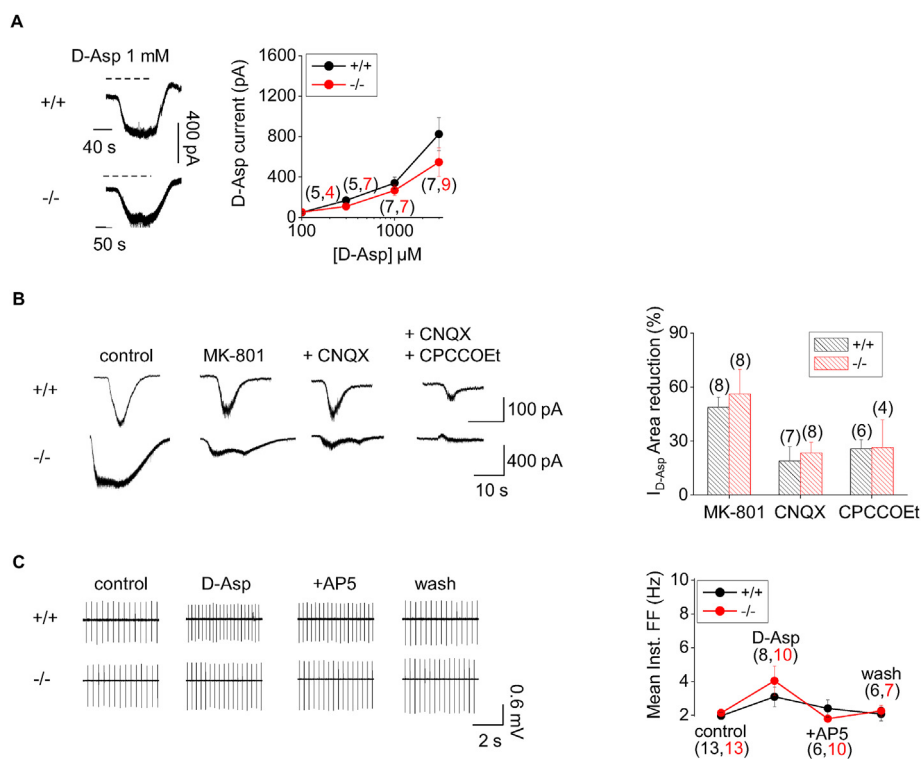
Fig. 2. Effects of D-Asp on membrane currents and spontaneous firing recorded from $Ddo^{+/+}$ and $Ddo^{-/-}$ DA neurons. (A) Example traces of inward currents from $Ddo^{+/+}$ (+/+) and $Ddo^{-/-}$ (−/−) cells elicited by bath application of D-Asp (1 mM; −60 mV). The dashed lines shows the duration of agonist application. The plot on the right shows the dose–response curves for D-Asp in the two genotypes. **(B)** Example traces from a single $Ddo^{+/+}$ (top row) and $Ddo^{-/-}$ neuron (bottom), of inward currents (−60 mV) evoked by pressure-ejected D-Asp (3 mM) in control conditions and during consecutive bath application of selective glutamate receptor antagonists (MK-801 30 μM, for NMDA receptors; CNQX 30 μM, for AMPA/kainate receptors and CPCCOEt 100 μM, for group I mGlu receptors); the bar graph shows the mean net D-Asp current area reduction due to each antagonist ($P > 0.05$ for all; unpaired t-test) for $Ddo^{+/+}$ (black) and $Ddo^{-/-}$ neurons (red). **(C)** Single-unit extracellular recordings of spontaneous firing (5 s stretches) from a $Ddo^{+/+}$ and a $Ddo^{-/-}$ neuron in control conditions and during bath application of the reported drugs (D-Asp 300 μM, D-Asp + AP5 100 μM). The plot shows pulled mean values of instantaneous firing frequency in each experimental condition ($P < 0.05$ for all treatments within each genotype, with paired t-test; between genotypes: $P > 0.05$, unpaired t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substantia nigra following DA receptor stimulation (Abarca et al., 1995). Also, early work showed that the brain levels of L-Asp, unlike those of D-Asp and NMDA, remain unchanged in $Ddo^{-/-}$ animals compared to controls (Errico et al., 2006; Huang et al., 2006). Thus, we used L-Asp in order to ascertain whether $Ddo^{-/-}$ DAergic neurons exhibited changes in glutamate receptor expression compared to $Ddo^{+/+}$ cells. We therefore tested the effects of L-Asp onto membrane currents and spontaneous firing of DAergic neurons (Fig. 3A, B, C). The whole-cell traces and dose–response curves in Fig. 3A indicate that L-Asp produces equivalent currents with those of D-Asp (Fig. 2A), suggesting similar sensitivity of DAergic neurons for the two agonists. Likewise, the effect of L-Asp was similar in $Ddo^{-/-}$ and $Ddo^{+/+}$ neurons at all concentrations tested (Fig. 3A).

To detect changes in the contribution of glutamate receptors in the currents activated by L-Asp, compared to D-Asp, in the two genotypes, we repeated the pharmacology experiments with glutamate receptor antagonists. L-Asp currents were gradually reduced with each added antagonist and completely blocked with all three together (Fig. 3B). However, surprisingly, the sensitivity for each antagonist differed between $Ddo^{-/-}$ and $Ddo^{+/+}$ mice: in $Ddo^{+/+}$ cells CNQX caused the biggest reduction, followed by MK-801 and CPCCOEt, suggesting that the majority of the L-Asp current is mediated by AMPA/kainate receptors. In $Ddo^{-/-}$ mice however, the NMDA receptor component was predominant. The tendency for stronger block of L-Asp currents by MK-801 in $Ddo^{-/-}$ mice (Fig. 3B), though not significant ($P = 0.071$), could suggest an increase in the population of NMDA receptors in $Ddo^{-/-}$ animals

(and/or a reduction of AMPA/kainate receptors). This hypothesis was more evident from extracellular recordings: L-Asp caused a much stronger effect on spontaneous firing in the $Ddo^{-/-}$ neurons compared to controls. In the example experiment in Fig. 3C the $Ddo^{-/-}$ cell fired at a higher frequency than the $Ddo^{+/+}$ neuron (4.41 Hz *versus* 2.67 Hz) and it depolarized more, as judged by the bigger reduction in spike amplitude. The $Ddo^{-/-}$ cell remained more excited throughout the experiment and did not completely recover to control firing levels even after the application of AP5 or after wash-out. This is evident from the cumulative plot in Fig. 3C, showing that, in $Ddo^{-/-}$ neurons, the mean firing frequency is significantly higher than in $Ddo^{+/+}$ cells, both during L-Asp and during L-Asp + AP5.

3.4. Selective NMDA receptor activation and surface expression of NMDA receptors in $Ddo^{+/+}$ and $Ddo^{-/-}$ DAergic neurons

The results reported so far point to the observation that $Ddo^{-/-}$ DAergic cells show enhanced NMDA receptor sensitivity, possibly due to increased expression of NMDA receptors. To investigate this point further we used NMDA, a selective NMDA receptor agonist, to study NMDA-induced currents by means of whole-cell recordings (−60 mV). Fig. 4A shows example traces from $Ddo^{+/+}$ and $Ddo^{-/-}$ cells using normal or Mg^{2+}-free solutions, to prevent the Mg^{2+}-induced NMDA receptor block. As expected, in Mg^{2+}-free conditions currents were almost 10-fold bigger. As seen from the data plots in Fig. 4B, irrespective of which solutions were used, mean NMDA currents were significantly larger in $Ddo^{-/-}$ cells (with
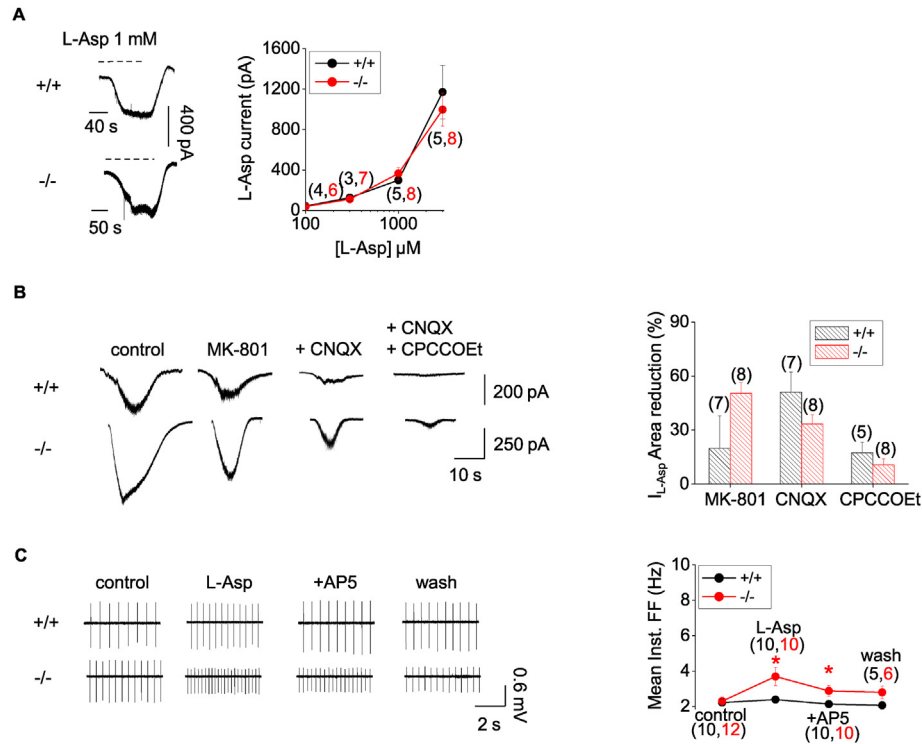
Fig. 3. Effects of L-Asp on membrane currents and spontaneous firing recorded from $Ddo^{+/+}$ and $Ddo^{-/-}$ DA neurons. (A) Examples of inward currents from $Ddo^{+/+}$ (+/+) and $Ddo^{-/-}$ (−/−) cells elicited by bath application of L-Asp (1 mM; −60 mV). The dashed lines shows the duration of agonist application. The plot on the right shows the dose−response curves for L-Asp in the two genotypes. (B) Example inward currents (−60 mV) from a $Ddo^{+/+}$ (top) and $Ddo^{-/-}$ neuron (bottom), evoked by pressure-ejected L-Asp (3 mM) in control conditions and during consecutive bath application of selective glutamate receptor antagonists (MK-801 30 μM, for NMDA receptors; CNQX 30 μM, for AMPA/kainate receptors and CPCCOEt 100 μM, for group I mGlu receptors); the bar graph shows the mean net L-Asp current area reduction due to each antagonist ($P > 0.05$ for all, unpaired t-test) for $Ddo^{+/+}$ (black) and $Ddo^{-/-}$ neurons (red). (C) Extracellular recordings of spontaneous firing (5 s stretches) from a $Ddo^{+/+}$ and a $Ddo^{-/-}$ neuron in control conditions and during bath application of the reported drugs (L-Asp 300 μM, L-Asp + AP5 100 μM). The plot shows the mean instantaneous firing frequency in each condition ($P < 0.05$ for all treatments within each genotype, paired t-test). Note that in $Ddo^{-/-}$ in the mean firing frequency is significantly higher both during L-Asp ($P = 0.026$) and during L-Asp + AP5 ($P = 0.041$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
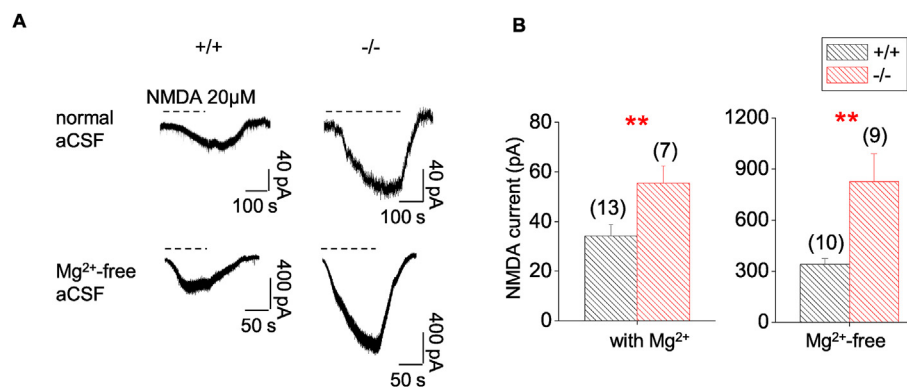

Fig. 4. Selective activation of NMDA receptors reveals enhanced membrane currents in $Ddo^{-/-}$ DA neurons. (A) Example inward currents (−60 mV) elicited by bath application of NMDA in normal aCSF (top) and Mg^{2+}-free aCSF (bottom). The dashed lines shows the duration of agonist application. Note the approximately 10-fold increase in the current amplitude in the absence of Mg^{2+}. (B) The plots show corresponding averaged data of NMDA current peak amplitudes in normal (left) and Mg^{2+}-free aCSF (right).

Mg^{2+}: $P = 0.007$; in Mg^{2+}-free: $P = 0.008$), providing a strong evidence that persistent elevation of D-Asp in the brain of $Ddo^{-/-}$ mice increases the NMDA receptor population in DAergic neurons.

Our electrophysiological data were confirmed by the analysis of surface levels of the GluN1, GluN2A and GluN2B subunits of NMDA receptors in the SNc of $Ddo^{+/+}$ and $Ddo^{-/-}$ mice using a biotinylation assay. We performed this assay in mice not older than P19 (P17–P19), as the levels of midbrain NMDA receptor subunits change during postnatal development and GluN2 proteins are

reduced in the adult brain (Albers et al., 1999; Brothwell et al., 2008; Pearlstein et al., 2015). Indeed, we found that in midbrain slices from $Ddo^{-/-}$ mice the surface and total levels of the GluN1 and GluN2A subunits were significantly increased compared to $Ddo^{+/+}$ slices (Fig. 5A, B). Although the GluN2B subunit is functionally important in dopaminergic neurons of the SNc (Brothwell et al., 2008; Pearlstein et al., 2015), no differences in this subunit were observed between the two genotypes. Similarly, no changes in AMPA receptor GluA1 subunit expression were detected.
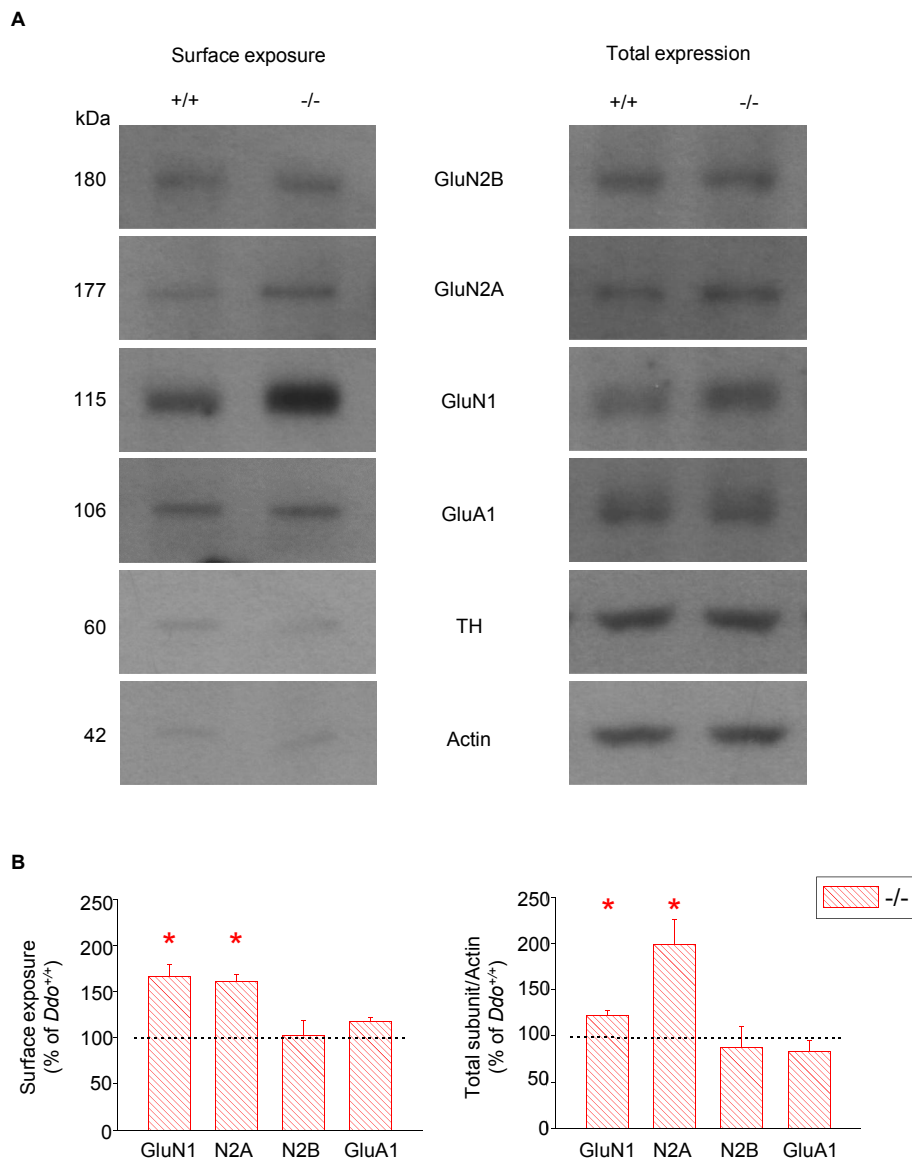
Fig. 5. NMDA receptor GluN1 and GluN2A subunit expression is increased in midbrain slices of $Ddo^{-/-}$ mice. **(A)** Representative immunoblots of biotinylated proteins (surface exposure, left) and total extracts (total protein, right) from $Ddo^{+/+}$ (+/+) and $Ddo^{-/-}$ (−/−) midbrain slices (n = 3). Actin was used as loading control in total extracts, whereas the absence of TH and actin in surface samples indicates the specificity of the biotinylation assay. **(B)** Histograms of densitometric quantification of changes in gray values, expressed as % of controls for subunits expressed in the cell surface (left; $P = 0.021$ for surface GluN1, $P = 0.004$ for GluN2A) and for total extracts (right; $P = 0.046$ for total GluN1, $P = 0.047$ for GluN2A).

3.5. Effects of D-Asp uptake block in $Ddo^{-/-}$ DAergic neurons

In view of the fact that NMDA receptors are increased on the surface of $Ddo^{-/-}$ neurons, it is unclear why the exogenous application of D-Asp did not yield any significant differences compared to control cells (unlike what we observed for L-Asp and NMDA). Thus, we tested if EAATs participate to the same extent in the uptake of D- and L-Asp in $Ddo^{-/-}$ and $Ddo^{+/+}$ cells, by using DL-TBOA, a competitive EAAT blocker. D- or L-Asp were pressure-injected near a patched cell to evoke inward currents before and after bath application of DL-TBOA. As expected, DL-TBOA increased the amplitude and the duration of the inward currents, in agreement with a reduced uptake of the two agonists (Fig. 6A). In $Ddo^{+/+}$ neurons the effect of DL-TBOA is similar for both the D- and the L-Asp currents, whereas in $Ddo^{-/-}$ cells the D-Asp current is more potentiated (Fig. 6B). Thus, it appears that in $Ddo^{-/-}$ cells the EAATs

are more efficient in uptaking D-Asp than L-Asp, providing an explanation as to why we fail to observe differences between $Ddo^{+/+}$ and $Ddo^{-/-}$ neurons when D-Asp is used. Of note, DL-TBOA had no effect in NMDA-induced currents (data not shown).

4. Discussion

We assessed for the first time the effects of D-Asp on midbrain SNc dopaminergic cells of wild-type and Ddo-knockout mice, and found that it can excite DAergic neurons by activating NMDA, AMPA and group I metabotropic glutamate receptors. Whilst persistent elevated endogenous D-Asp in the $Ddo^{-/-}$ mouse does not change the intrinsic electrophysiological properties of DAergic neurons, $Ddo^{-/-}$ cells show larger NMDA currents that reflect an increased surface expression of GluN1- and GluN2A-containing NMDA receptors.
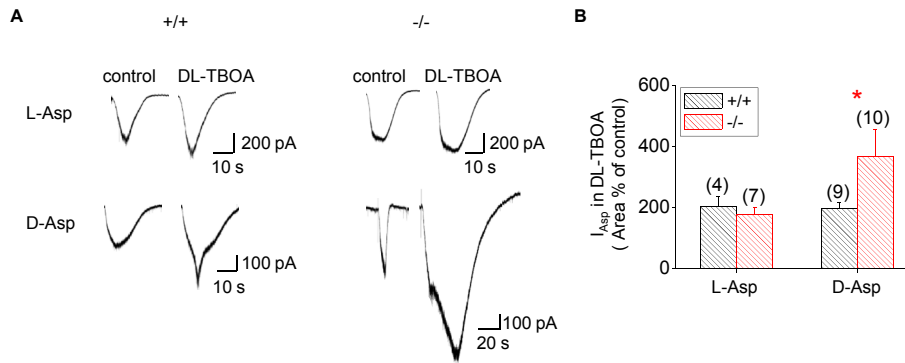
Fig. 6. Inhibition of Na^+-dependent excitatory amino-acid transporters potentiates D-Asp currents in $Ddo^{-/-}$ neurons. (A) Example traces of inward currents (-60 mV) elicited by L- or D-Asp (3 mM) pressure-ejected near a $Ddo^{+/+}$ ($+/+$) or $Ddo^{-/-}$ ($-/-$) cell in control conditions and during bath application of DL-TBOA (100 μM). **(B)** Pulled data showing mean values of current areas, elicited by L- and D-Asp, in the presence of DL-TBOA ($P < 0.05$ for all groups in respect to the control condition, paired t-test; between genotypes: $P > 0.05$ for L-Asp, $P = 0.050$ for D-Asp, unpaired t-test).

We also found that the endogenous agonist L-Asp excites control DAergic cells by mainly activating AMPA/kainate type ionotropic receptors but also NMDA and group I metabotropic receptors.

Finally, we present evidence that EAATs may uptake D-Asp more efficiently than L-Asp in $Ddo^{-/-}$ mice.

4.1. Glutamate receptor subtypes contribute at different extent in D- and L-Asp induced currents

D-Asp and L-Asp excite DAergic cells by producing comparable concentration-dependent inward currents. Selective glutamate antagonists showed that the D-Asp current is mediated mainly by NMDA receptors in both $Ddo^{+/+}$ and $Ddo^{-/-}$ DA neurons, but a significant contribution of AMPA/kainate and group I mGlu receptors is also present. On the other hand, L-Asp activates mainly AMPA/kainate receptors in $Ddo^{+/+}$ DA neurons. Irrespective of the agonist used, the NMDA receptor component is always prevalent in $Ddo^{-/-}$ cells.

For D-Asp, a prominent NMDA receptor-mediated component was already reported in the prefrontal cortical (Errico et al., 2014), striatal (Errico et al., 2008b) and hippocampal (Errico et al., 2008a; Kiskin et al., 1990) neurons. Moreover, in agreement with our data, D-Asp activates mGlu$_5$ receptors in neonatal cortical and hippocampal neurons (Molinaro et al., 2010). However, these studies did not report activation of AMPA/kainate receptors by D-Asp, as we observed in nigral neurons. Also, D-Asp (and NMDA), but not L-Asp, can inhibit AMPA receptor-mediated kainate and L-glutamate currents in isolated rat hippocampal neurons and is unable to activate recombinant homomeric AMPA receptors (Gong et al., 2005). These studies conflict with ours; our results, showing AMPA receptor activation, suggest that neuronal responses to D-Asp may depend on the receptor isoform expressed on each cell type. In support of this, we previously showed that another natural amino-acid, β-N-methylamino-L-alanine (BMAA) activates different glutamate receptors in different brain areas: BMAA activates metabotropic/AMPA receptors in nigral DAergic neurons (Cucchiaroni et al., 2010), NMDA receptors in cortical neurons (Lobner et al., 2007; Weiss et al., 1989) and AMPA/kainate receptors in spinal motoneurons (Rao et al., 2006).

It is also possible that part of the D-Asp responses we observed in $Ddo^{-/-}$ cells result from modulations of presynaptic glutamate release by the elevated D-Asp. Accordingly, *in vivo* microdialysis in freely-moving $Ddo^{-/-}$ mice showed increased basal extracellular glutamate levels in the hippocampus, whereas transient D-Asp injections in wild-types trigger glutamate release in the prefrontal cortex (Cristino et al., 2015). It would be interesting to assess the possible effect of D-Asp on presynaptic mechanisms by using electrophysiological techniques, although the strong postsynaptic component activated by this agonist makes such experiments challenging.

4.2. Persistent D-Asp elevation does not alter intrinsic properties of Ddo$^{-/-}$ DAergic neurons

The spontaneous firing frequency, R_m (at -60 mV), C_m, and I_h amplitude of young adult $Ddo^{-/-}$ neurons are similar to controls, despite a reported increase of D-Asp and NMDA brain levels (Errico et al., 2006; Huang et al., 2006). The DA sensitivity is also unaltered. Thus, at this developmental stage elevated D-Asp levels, that provide persistent hyper-stimulation of glutamatergic transmission, do not appear to alter the basic electrophysiological function or the viability of DAergic cells. Our results suggest that in *in vitro* SNc preparations there are insufficient D-Asp levels in the extracellular space to alter the firing of DAergic neurons. In fact, it is noteworthy that, in an *in vivo* study, Schweimer et al. (2014) reported increased burst firing of midbrain DAergic neurons in D-amino acid oxidase knockout mice, as a consequence of increased extracellular D-serine that could activate NMDA receptors.

The fact that exogenous D-Asp application has similar effects in the two genotypes appears be due to a more efficient mechanism of D-Asp (but not L-Asp) removal from the extracellular space in $Ddo^{-/-}$ neurons, revealed by the DL-TBOA experiments. The mechanism by which the EAATs are more efficient in uptaking D-Asp in $Ddo^{-/-}$ DAergic cells is not clear yet. It does not appear to be stereospecific; the five known transporters (EAAT1-5) are stereospecific for glutamate but cannot discriminate between aspartate enantiomers (Palacín et al., 1998) and, as seen in $Ddo^{+/+}$ animals, the effect of DL-TBOA on L-Asp and D-Asp currents is identical. The EAATs have similar affinity for both D-Asp and L-Asp (Kanai and Hediger, 1992; Masson et al., 1999) ruling out the possibility that our results are due to increased affinity of the $Ddo^{-/-}$ transporters for D-Asp. It might be that in $Ddo^{-/-}$ mice there is a change in transporter numbers; in fact, some pharmacological reagents, such as amphetamines, can reduce the surface expression of EAAT3 by endocytosis (Underhill et al., 2014). However, such a change in transporter numbers would have similar effects for both D- and L-Asp currents in $Ddo^{-/-}$ mice, but our data show specificity for D-Asp only. Our results point to the presence of increased basal extracellular D-Asp in $Ddo^{-/-}$ mice and near-saturating removal by

EAATs. In the presence of DL-TBOA, when the uptake is blocked, the effect of this unremoved basal D-Asp could add to the effect of exogenous D-Asp we apply, to significantly potentiate D-Asp currents, as we observed. However, the enhanced extracellular D-Asp levels are not *per se* sufficient to affect the overall excitability of $Ddo^{-/-}$ cells, as we noticed during the extracellular recordings. Notably, EAAT3 has high affinity (μmol range) for its substrates in order to protect cells from glutamate-induced excitotoxicity (Masson et al., 1999). Thus, the lack of differences in the spontaneous firing activity between $Ddo^{-/-}$ and $Ddo^{+/+}$ cells can be attributed to the more efficient uptake of D-Asp by the EAATs.

4.3. Persistent D-Asp elevation in $Ddo^{-/-}$ DAergic neurons increases NMDA receptor responses and GluN1 and GluN2A subunit expression

Although the L-Asp dose−response curves from the two genotypes showed identical currents, the pharmacological analysis revealed a trend for an increase in the MK-801-sensitive component in $Ddo^{-/-}$ neurons. More importantly, the $Ddo^{-/-}$ spontaneous firing was significantly more sensitive to L-Asp compared to control cells. We hypothesized that these differences reflect a shift towards more functional NMDA receptors in $Ddo^{-/-}$ cells. In agreement with this, exogenous NMDA induced larger inward currents in $Ddo^{-/-}$ neurons. We can speculate that an increased glutamatergic drive to $Ddo^{-/-}$ DAergic neurons *in vivo* may cause augmented dopamine levels in target areas.

Our hypothesis was further confirmed by biotinylation indicating a significant increase in the GluN1 and GluN2A subunit total and surface expression in developing $Ddo^{-/-}$ neurons. Surprisingly, we did not observe significant differences between $Ddo^{-/-}$ and control mice in the total and surface levels of the GluN2B subunit. Under conditions of physiological midbrain development, the GluN2A subunit levels are reduced with aging and functional somatic NMDA receptors are formed mainly by GluN2B and GluN2D subunits in combinations with GluN1 (Brothwell et al., 2008; Pearlstein et al., 2015). Our data suggest that, under conditions of prolonged D-Asp during development, the increased levels of the GluN2A subunit could substitute for GluN2B for the formation of functional NMDA receptors in $Ddo^{-/-}$ dopaminergic neurons, thus explaining the larger inward currents we recorded in these cells. A replacement of GluN2B by GluN2A is also seen in cortical and hippocampal pyramidal neurons (Williams et al., 1993; Flint et al., 1997; Tovar and Westbrook, 1999).

4.4. Conclusions and functional considerations

Excessive stimulation of glutamate receptors on DAergic cells can cause excitotoxicity (Surmeier et al., 2010) while striatal hyper-dopaminergia can produce excessive movements (dyskinesia, chorea and tics) and facilitate compulsive behaviour and drug seeking (Gainetdinov et al., 1999). Furthermore, elevated dopamine synthesis capacity in nigral dopamine neurons, as well as in their striatal terminals, has been linked to symptom severity in schizophrenic patients (Howes et al., 2011). In this manuscript we show that D-Asp can activate different glutamate receptors in DAergic neurons. However, under conditions of chronic D-Asp elevation (as represented by the $Ddo^{-/-}$ mice) an enhanced uptake mechanism can prevent D-Asp-induced over-excitation of the DAergic cells and the consequent hyper-dopaminergia in target zones. More experiments are necessary in the future to comprehend better the mechanisms occurring at the level of the EAATs and how D-Asp transport is regulated under physiological conditions and during long-term increases in D-Asp levels.

Author contribution

- All authors are accountable for all aspects of accuracy and integrity of the work performed, have made intellectual contributions to the data analysis and interpretation, contributed to manuscript preparation and approved the final version to be submitted.
- P.K., A.L, A.C. and E.G. designed and performed the electrophysiology experiments and analysed data.
- A.N. and M.D.A. designed and performed the biotylination/immunoblottin experiments and analysed data.
- A.U., M.D.A., N.B.M. and I.C. conceived the study and designed experiments.
- A.U. and F.E. contributed the knockout mice for the study.

Conflicts of interest

The authors declare no competing interests.

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