

## RESEARCH PAPER

# Enantioselective inhibition of D-serine transport by (S)-ketamine

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### Received

18 December 2014

### Revised

26 June 2015

### Accepted

28 June 2015

## BACKGROUND AND PURPOSE

Patients with major depressive disorder receiving racemic ketamine, (*R,S*)-ketamine, experience transient increases in Clinician-Administered Dissociative States Scale scores and a coincident drop in plasma D-serine levels. The results suggest that (*R,S*)-ketamine produces an immediate, concentration-dependent pharmacological effect on D-serine plasma concentrations. One potential source of this effect is (*R,S*)-ketamine-induced inhibition of the transporter ASCT2, which regulates intracellular D-serine concentrations. In this study, we tested this hypothesis by examining the effect of (*S*)- and (*R*)-ketamine on ASCT2-mediated transport of D-serine in PC-12 and 1321N1 cells and primary neuronal cells in culture.

## EXPERIMENTAL APPROACH

Intracellular and extracellular D-serine levels were determined using capillary electrophoresis–laser-induced fluorescence and liquid chromatography–mass spectrometry respectively. Expression of ASCT2, Asc-1 and serine racemase was determined utilizing Western blotting.

## KEY RESULTS

(*S*)-Ketamine produced a concentration-dependent increase in intracellular D-serine and reduced extracellular D-serine accumulation. In contrast, (*R*)-ketamine decreased both intracellular and extracellular D-serine levels. The ASCT2 inhibitor, benzyl-D-serine (BDS), and ASCT2 gene knockdown mimicked the action of (*S*)-ketamine on D-serine in PC-12 cells, while the Asc-1 agonist D-isoleucine reduced intracellular D-serine and increased extracellular D-serine accumulation. This response to D-isoleucine was not affected by BDS or (*S*)-ketamine. Primary cultures of rat neuronal cells expressed ASCT2 and were responsive to (*S*)-ketamine and BDS. (*S*)- and (*R*)-ketamine increased the expression of monomeric serine racemase in all the cells studied, with (*S*)-ketamine having the greatest effect.

## CONCLUSIONS AND IMPLICATIONS

(*S*)-Ketamine decreased cellular export of D-serine via selective inhibition of ASCT2, and this could represent a possible source of dissociative effects observed with (*R,S*)-ketamine.

## Abbreviations

BDS, benzyl-D-serine; CADSS, Clinician-Administered Dissociative States Scale; D-IL, D-isoleucine; Ket, (*R,S*)-ketamine; MADRS, Montgomery–Åsberg Depression Rating Scale; MDD, major depressive disorder; m-SR, monomeric serine racemase; R-Ket, (*R*)-ketamine; S-Ket, (*S*)-ketamine

## Tables of Links

TARGETS	
<b>Ligand-gated ion channels<sup>a</sup></b>	<b>Transporters<sup>b</sup></b>
nAChR	Asc-1 (SLC7A10)
NMDA receptor	ASCT1
	ASCT2

LIGANDS
D-serine
Ketamine
Nicotine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (<sup>a,b</sup>Alexander *et al.*, 2013a,b).

## Introduction

(*R,S*)-Ketamine is a phencyclidine derivative that produces rapid and short-lived anaesthesia with a wide margin of clinical safety (Domino, 2010; Hirota and Lambert, 2011); however, disturbing emergence reactions and dissociative and hallucinogenic effects have been reported following the use of (*R,S*)-ketamine (White *et al.*, 1982). Recent studies have shown that (*R,S*)-ketamine is also an effective antidepressant agent, as illustrated by the work of Zarate *et al.* (2006), in which a single sub-anaesthetic dose of the drug produced rapid antidepressant responses in patients with treatment-resistant major depressive disorder (MDD). While the use of (*R,S*)-ketamine in the treatment of MDD and bipolar depression is an effective and increasingly popular therapeutic approach, its administration is also accompanied by dissociative side effects as measured by an increase in the Clinician-Administered Dissociative States Scale (CADSS) (Pomarol-Clotet *et al.*, 2006; Zarate *et al.*, 2012; Luckenbaugh *et al.*, 2014). In the study by Luckenbaugh *et al.* (2014), the maximum increases in CADSS scores were observed at the end of a 40 min infusion of (*R,S*)-ketamine (0.5 mg·kg<sup>-1</sup>), which was followed by a rapid decline to below baseline at the 80 min sampling point. Moreover, a significant negative correlation was found between the magnitude of the increase in CADSS scores at 40 min and the (*R,S*)-ketamine-associated antidepressant effect at 230 min, determined as % improvement in the 17-item Hamilton Depression Rating Scale (Luckenbaugh *et al.*, 2014).

We have recently re-analysed a subset of the MDD patients from the study of Luckenbaugh *et al.* (2014), and demonstrated that the % change in the Montgomery-Åsberg Depression Rating Scale (MADRS) scores between baseline and 230 min after initiation of the study segregates the patients into responders and non-responders to (*R,S*)-ketamine antidepressant therapy, with response defined as a ≥50% decrease in the MADRS score (Moaddel *et al.*, 2015). Baseline plasma concentrations of D-serine, a key NMDA receptor co-agonist, were compared with the antidepressant response to (*R,S*)-ketamine treatment and were found to be significantly lower in responders than non-responders (Moaddel *et al.*, 2015). In addition, there was a significant relationship between baseline D-serine plasma concentrations and percentage change in MADRS.

The alanine-serine-cysteine transporter 2 (ASCT2) and neutral amino acid transporter Asc-1 are involved in the cellular uptake and release of D-serine (Rosenberg *et al.*, 2013; receptor and transporter nomenclature follows Alexander *et al.*, 2013b). The first objective of the current study was to determine the effect of (*R,S*)-ketamine on the intracellular and extracellular concentrations of D-serine in PC-12 pheochromocytoma cells, 1321N1 astrocytoma cells and primary rat neuronal cells, and to examine the role that ASCT2 and Asc-1 have in mediating (*R,S*)-ketamine responsiveness. The immortalized cell lines were chosen based upon previous data showing that incubation with the (*R,S*)-ketamine metabolite, (*R,S*)-dehydronorketamine, decreased the intracellular D-serine concentration in both cell lines (Singh *et al.*, 2013) and primary neuronal cells were studied based upon the report that these cells are a major source of D-serine (Kartvelishvili *et al.*, 2006). Of significance, (*R,S*)-ketamine is a chiral molecule existing as (*S*)-ketamine and (*R*)-ketamine enantiomers, with different pharmacological properties (Kohrs and Durieux, 1998; Domino, 2010; Hirota and Lambert, 2011). Moreover, (*R*)-ketamine exhibits a more potent and longer lasting antidepressant effect in mice than (*S*)-ketamine (Zhang *et al.*, 2014). For these reasons, the experiments were designed to investigate the effect of (*S*)-ketamine and (*R*)-ketamine on D-serine synthesis and transport in immortalized cell lines and primary rat neuronal cells.

## Methods

### Cell lines

The PC-12 pheochromocytoma cell line derived from rat adrenal medulla was obtained from American Type Culture Collection (Manassas, VA, USA). The human-derived 1321N1 astrocytoma cell line was obtained from European Collection of Cell Cultures (Sigma-Aldrich). DMEM with glutamine, RPMI-1640, trypsin solution, PBS, FBS, sodium pyruvate (0.1 M), L-glutamine (0.2 M) and penicillin/streptomycin solution (containing 10 000 u·mL<sup>-1</sup> penicillin and 10 000 µg·mL<sup>-1</sup> streptomycin) were obtained from Quality Biological (Gaithersburg, MD, USA), heat-inactivated horse serum was purchased from Biosource (Rockville, MD, USA) and HEPES buffer (1 M, pH 7.4) was obtained from Mediatech, Inc. (Manassas, VA, USA). The PC-12 cells were main-

tained in RPMI-1640 supplemented with 1 mM HEPES, pH 7.4, 10% horse serum, 5% FBS, 1% sodium pyruvate, 5% L-glutamine and 1% penicillin/streptomycin, and the 1321N1 cells were maintained in DMEM with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin.

### Primary neuronal cultures

Cultures of cortical and hippocampal neurons were prepared from embryonic day 18 rat brains, as described previously (Mattson *et al.*, 1988). Dissociated neurons were plated on 60 × 15 mm tissue culture plates coated with polyethyleneimine and grown in neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA). All experimental treatments were performed in the same media on 7-day-old cultures.

### Determination of intracellular and extracellular D-serine concentrations

Intracellular D-serine concentrations were measured using a previously described and validated capillary electrophoresis-laser-induced fluorescence (CE-LIF) method using a P/ACE MDQ system equipped with a LIF detector (Beckman Instruments, Fullerton, CA, USA) (Singh *et al.*, 2012). The extracellular concentrations of D-serine were determined using a previously reported assay employing liquid chromatography with mass spectrometric detection (Xie *et al.*, 2014), which was optimized for use in this study. In brief, the chromatographic experiments were carried out on a Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA) coupled to a 5500 QTRAP triple quadrupole mass spectrometer equipped with a Turbo V electrospray ionization source (AB Sciex, Concord, ON, Canada). A 100 µL aliquot of the incubation media was combined with 20 µL aliquot of IS (10 µM D-arginine in acetone) and 400 µL acetone, and then centrifuged at 13 000× *g* for 10 min at 4°C. A 400 µL aliquot of the supernatant was derivatized with 300 µL of a 1 mM solution of (R)-1-Boc-2-piperidinecarbonyl chloride. The solution was evaporated to dryness and the residue was dissolved in 100 µL of methanol/water (10:90, *v v*<sup>-1</sup>) before being transferred to the autosampler for analysis. (R)-1-Boc-2-piperidinecarbonyl chloride was prepared by mixing 28 µL of a 72 mM triethylamine solution in acetone with 2 mM (R)-1-Boc-2-piperidineacetic acid in 500 µL of acetone and 500 µL of a 1 mM cyanuric chloride solution in acetone. This mixture was incubated by stirring at 1000 rpm for 3 h at 28°C. The reaction was terminated on ice and the product, (R)-1-Boc-2-piperidinecarbonyl chloride, was stored at -70°C before use. Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA) protected with an Agilent C18 guard column at room temperature. The mobile phase consisted of water with 0.3% trifluoroacetic acid (elute A) and methanol with 0.3% trifluoroacetic acid (elute B). The gradient eluent was set at a flow rate of 0.4 mL·min<sup>-1</sup> and programmed as follows: 0–15 min, 5–9% B; 15–22 min, 15% B; 22–25 min, 5% B. Positive electrospray ionization data were acquired using multiple reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray), collision energy and ion spray voltage were 550°C,

20 psi, 45 psi, 80 psi, 15 V and 4500 V respectively. The TIS compound parameter settings for declustering potential, entrance potential and collision cell exit potential were 80, 10 and 10 V respectively. The standards were characterized using the following MRM ion transitions: D-Ser derivatization product (*m/z* 231.5–106.1) and D-arginine derivatization product (*m/z* 300.4–175.0).

### Western blotting

PC-12 and 1321N1 cell lines and primary rat neuronal cells were lysed in RIPA containing EDTA and EGTA (Boston Bio-Products, Ashland, MA, USA) and supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail sets I and II (EMD Millipore, Billerica, MA, USA). Protein concentrations were determined in the clarified lysates using the bicinchoninic acid reagent (Thermo Fisher Scientific, Waltham, MA, USA). Proteins (20 µg per well) were separated on 4–12% precast gels (Invitrogen) using SDS-PAGE under reducing conditions and then electrophoretically transferred onto PVDF membranes (Invitrogen). Western blotting experiments were performed according to standard methods, which involved a blocking step in 5% non-fat milk/0.1% Tween-20 in PBS and incubation with the primary antibody of interest, followed by incubation with a secondary antibody conjugated with the enzyme HRP. The detection of immunoreactive bands was performed using the ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA). The quantification of bands was done by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalization to β-actin. The primary antibodies for the determination of monomeric and dimeric serine racemase (Cat. No. ab45434) and β-actin (Cat. No. ab6276) were purchased from Abcam, Inc. (Cambridge, MA, USA), whereas the primary antibodies raised against the Na<sup>+</sup>-dependent alanine-serine-cysteine transporter 1 (ASCT1) (Cat. No. sc-134846), ASCT2 (Cat. No. sc-130963) and Na<sup>+</sup>-independent alanine-serine-cysteine transporter 1 (Asc1) (Cat. No. sc-292032) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies were used at a dilution recommended by the manufacturer.

### ASCT2 silencing in PC-12 cells and treatment with (S)-ketamine and BDS

The siRNA oligos targeting ASCT2 (SMARTpool: siGENOME Rat Slc1a5 siRNA) were obtained from GE Dharmacon (Lafayette, CO, USA). PC-12 cells were seeded into 6-well plates (200 000 cells per well) and cultured for 24 h. Adherent cells were subjected to siRNA transfection using lipofectamine-RNAiMAX (Invitrogen) according to the manufacturer's recommendations. The final concentration of siRNA in each well was 100 nM. After 24 h of transfection, culture medium containing 10% serum was added and the expression of ASCT2 determined by Western blot. In the next series of experiments, ASCT2-silenced PC-12 cells were incubated for an additional 36 h with S-ketamine (0.25 µM) and BDS (50 µM). The intracellular and extracellular D-serine levels were determined using three replicate dishes. This experiment was repeated in three independent cell preparations (*n* = 3).

### *Effects of (R)-ketamine, (S)-ketamine and BDS on intracellular and extracellular D-serine levels and expression of serine racemase in PC-12 and 1321N1 cells*

Cells were seeded on 100 × 20 mm tissue culture plates and maintained at 37°C under humidified 5% CO<sub>2</sub> in air until they reached >70% confluence. The original medium was replaced with a medium containing the test compounds and the plates were incubated for an additional 36 h, unless otherwise indicated. The medium was removed, and the cells were collected for analysis. (R)-ketamine and (S)-ketamine (0–10 μM), BDS (0–1000 μM) or the combination (S)-ketamine (0.25 μM) + BDS (50 μM) were tested. Intracellular and extracellular D-serine levels were determined, as well as the expression of monomeric and dimeric serine racemase (m-SR, d-SR). The intracellular and extracellular D-serine levels were determined in triplicate dishes, while the determination of serine racemase protein expression was carried out on one set of dishes. Both analyses were repeated in three independent cell cultures (*n* = 3).

### *Effects of (R)-ketamine, (S)-ketamine and BDS on intracellular and extracellular D-serine levels in primary rat neuronal cells*

Cells were seeded on 60 × 15 mm tissue culture plates and maintained at 37°C under humidified 5% CO<sub>2</sub> in air for 7 days, during which time neurons grew axons and dendrites, and formed synapses. The original medium was replaced with a medium containing the test compounds and the plates were incubated for an additional 36 h, unless otherwise indicated. The medium was removed, and the cells were collected for analysis. In the first series of experiments, the effects of (R)-ketamine (1.0 μM) and (S)-ketamine (0.5 μM) were determined. In the second series of experiment, (S)-ketamine (0.5 μM) and BDS (50 μM), or the combination (S)-ketamine (0.5 μM) + BDS (50 μM) were tested. The intracellular and extracellular D-serine levels were determined in triplicate dishes.

### *Effects of D-isoleucine and D-isoleucine plus (S)-ketamine on the intracellular and extracellular D-serine levels and expression of serine racemase in PC-12 cells*

Cells were seeded on 100 × 20 mm tissue culture plates and maintained at 37°C under humidified 5% CO<sub>2</sub> in air until they reached >70% confluence. The original medium was replaced with the medium containing the test compounds and the plates were incubated for an additional 36 h. The medium was removed, and the cells were collected for analysis. In the first series of experiments, D-isoleucine concentrations ranged from 0 to 2000 μM. In the second series of experiment, incubations were conducted using D-isoleucine (200 μM), (S)-ketamine (0.6 μM) and D-isoleucine (200 μM) plus (S)-ketamine (0–10 μM). Intracellular and extracellular D-serine levels were determined, as well as the expression of monomeric serine racemase (m-SR) and d-SR. The intracellular and extracellular D-serine levels were determined in triplicate dishes, while the determination of serine racemase

protein expression was carried out on one set of dishes. Both analyses were repeated in three independent cell cultures (*n* = 3).

### *Statistical analysis*

Data are presented as average relative change ± SD. All statistical analyses were performed using one-way ANOVA and Dunnett's test for *post hoc* multiple comparisons. GraphPad Prism 4 software package running on a personal computer (GraphPad Software, La Jolla, CA, USA) was used to carry out statistical analyses. *P*-values of 0.05 or less were considered statistically significant. The observed changes in the D-serine levels was used to determine EC<sub>50</sub> and/or IC<sub>50</sub> sigmoidal dose-response curves using the 'nonlinear regression (curve fit)' model contained within the Prism 4 software package.

### *Materials*

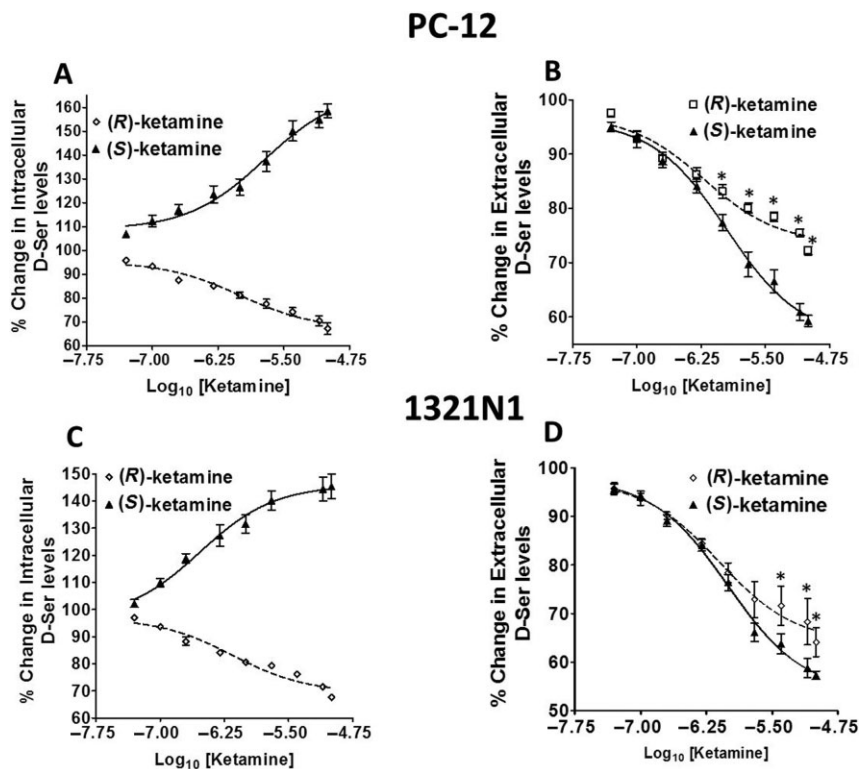
(R)-Ketamine and (S)-ketamine were prepared as previously described (Moaddel *et al.*, 2010). D-Serine, benzyl-D-serine (BDS), D-arginine, D-isoleucine, acetonitrile, methanol, trifluoroacetic acid, triethylamine, cyanuric chloride and (R)-1-Boc-2-piperidineacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). All other chemicals used were of analytical grade.

## **Results**

The effects of the test agents on D-serine concentration in the intracellular compartment and the incubation medium (extracellular) are presented as % change relative to the initial amount of D-serine in the respective milieu. Significant differences between data sets are set at *P* < 0.05, unless stated otherwise.

Incubation of PC-12 cells with (S)-ketamine (0–10 μM) produced a concentration-dependent increase in intracellular D-serine levels, with a maximum increase of 58.6 ± 7.3% and an EC<sub>50</sub> value of 0.82 ± 0.29 μM (Figure 1A). There was a reciprocal significant decrease in extracellular D-serine levels, with a maximum decrease of 40.7 ± 3.1% and a calculated IC<sub>50</sub> value of 0.76 ± 0.13 μM (Figure 1B). Unlike the effect observed with (S)-ketamine, incubation of PC-12 cells with (R)-ketamine (0–10 μM) produced a significant concentration-dependent decrease in the intracellular concentration of D-serine with a maximum decrease of 33.73 ± 8.1% and a calculated IC<sub>50</sub> value of 0.94 ± 0.16 μM (Figure 1A). There was a concomitant significant decrease in the extracellular D-serine concentration with a maximum effect of 27.8 ± 2.3% and a calculated IC<sub>50</sub> value of 0.70 ± 0.10 μM (Figure 1B).

Incubation of 1321N1 cells with (S)-ketamine and (R)-ketamine produced similar significant effects on the intracellular and extracellular concentrations of D-serine. (S)-Ketamine produced an increase in the intracellular D-serine concentration of 45.6 ± 8.9%, while (R)-ketamine produced a 32.3 ± 1.0% decrease with calculated EC<sub>50</sub> and IC<sub>50</sub> values of 0.46 ± 0.25 and 0.75 ± 0.27 μM, respectively (Figure 1C). Incubation with (S)-ketamine and (R)-ketamine resulted in decreased extracellular D-serine concentrations of 42.7 ± 2.1



**Figure 1**

Effects of ketamine enantiomers on the cellular partitioning of D-serine (D-Ser) in PC-12 and 1321N1 cells. PC-12 (A, B) and 1321N1 (C, D) cells were incubated with increasing concentrations of (*R*)-ketamine (0–10  $\mu$ M) or (*S*)-ketamine (0–10  $\mu$ M) for 36 h followed by the determination of intracellular (panels A and C) and extracellular (panels B and D) D-serine levels. The  $EC_{50}$  and  $IC_{50}$  values were calculated and are presented in the Results section. The  $EC_{50}$  and  $IC_{50}$  values for (*S*)-ketamine, based upon the intracellular and extracellular D-serine levels in PC12 cells, were  $0.82 \pm 0.29$  and  $0.76 \pm 0.13$   $\mu$ M respectively. In (*S*)-ketamine-treated 1321N1 cells, the  $EC_{50}$  and  $IC_{50}$  values were  $0.46 \pm 0.25$  and  $0.57 \pm 0.32$   $\mu$ M respectively.  $IC_{50}$  values for (*R*)-ketamine, based upon the extracellular and intracellular D-Ser levels in PC12 cells, were  $0.94 \pm 0.16$  and  $0.70 \pm 0.10$   $\mu$ M, whereas in 1321N1 cells, the  $IC_{50}$  values were  $0.75 \pm 0.27$  and  $0.88 \pm 0.25$   $\mu$ M. Data represent the average  $\pm$  SD of three independent experiments, where  $*P < 0.05$ .

and  $38.3 \pm 5.9\%$ , with  $IC_{50}$  values of  $0.57 \pm 0.32$  and  $0.88 \pm 0.25$   $\mu$ M, respectively (Figure 1D).

The incubation of primary rat neuronal cells obtained from cultures of cortical and hippocampal neurons with (*S*)-ketamine (0.5  $\mu$ M) and (*R*)-ketamine (1.0  $\mu$ M) produced the same qualitative and significant changes in the intracellular and extracellular concentrations of D-serine as observed in the immortalized cell lines. (*S*)-Ketamine increased the intracellular D-serine concentration by  $18.7 \pm 2.5\%$  in the cortex-derived cells and by  $19.8 \pm 2.6\%$  in the hippocampus-derived cells, while (*R*)-ketamine decreased the amount of intracellular D-serine by  $32.3 \pm 1.0\%$  (cortex-derived) and  $32.3 \pm 1.0\%$  (hippocampus-derived), respectively (Table 1). Incubation with (*S*)-ketamine and (*R*)-ketamine resulted in the lowering of the extracellular D-serine levels of  $18.6 \pm 1.9$  and  $16.4 \pm 3.2\%$  in the cortex-derived cells, and  $18.7 \pm 2.0$  and  $18.6 \pm 1.9\%$  decreases in the hippocampus-derived cells (Table 1).

The data demonstrate that there is a significant enantioselective difference in the effect of (*S*)-ketamine and (*R*)-ketamine on the intracellular D-serine concentration in both

PC-12 and 1321N1 cell lines. Significance was reached at (*S*)-ketamine and (*R*)-ketamine concentrations of 0.100  $\mu$ M in both cell lines (Figure 1A,C). The same enantioselective property was observed in the cortex-derived and hippocampus-derived cells (Table 1). (*S*)-Ketamine and (*R*)-ketamine also produced distinct effects on the extracellular D-serine levels in both PC-12 and 1321N1 cells. However, these effects did not reach significance when concentrations of (*S*)-ketamine and (*R*)-ketamine lower than 2.0  $\mu$ M were used in PC-12 cells, while a concentration of 4.0  $\mu$ M was required to produce a significant difference between the enantiomers in 1321N1 cells (Figure 1B,D). In the studies with the cortex-derived and hippocampus-derived cells, (*S*)-ketamine (0.5  $\mu$ M) and (*R*)-ketamine (1.0  $\mu$ M) did not result in a significant enantioselective difference in the ketamine-associated decrease in the amount of extracellular D-serine (Table 1).

The expression of ASCT2 and Asc-1 was established in PC-12, 1321N1, cortex-derived and hippocampus-derived cells by Western blotting, using lysate from the C6 glioblastoma cell line as positive control (Sikka *et al.*, 2010) (Figure 2). The data established the presence of these transporters, as

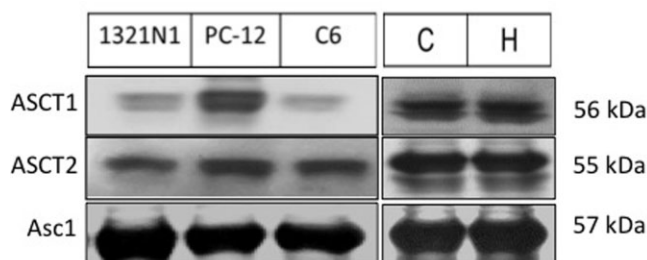
**Table 1**

The change in intracellular and extracellular D-serine concentrations in primary neuronal cells derived from rat cortex and hippocampus produced by incubation with *R*-ketamine, *S*-ketamine, BDS and BDS plus *S*-ketamine

Samples	% change in intracellular D-serine levels		% change in extracellular D-serine levels	
	Primary neuronal cells derived from cortex	Primary neuronal cells derived from hippocampus	Primary neuronal cells derived from cortex	Primary neuronal cells derived from hippocampus
Control	100.00	100.00	100.00	100.00
( <i>R</i> )-Ket (1 $\mu$ M)	-17.7 $\pm$ 2.5%*	-19.8 $\pm$ 2.6%*	-16.4 $\pm$ 3.2%*	-18.6 $\pm$ 1.9%*
( <i>S</i> )-Ket (0.5 $\mu$ M)	+22.1 $\pm$ 3.5%*	+24.6 $\pm$ 2.6%*	-19.5 $\pm$ 1.9%*	-18.7 $\pm$ 2.0%*
BDS (50 $\mu$ M)	+21.6 $\pm$ 1.9%*	+22.2 $\pm$ 1.9%*	-18.5 $\pm$ 2.8%*	-16.5 $\pm$ 1.6%*
BDS + ( <i>S</i> )-Ket	+47.2 $\pm$ 5.1%*	+45.3 $\pm$ 3.4%*	-44.1 $\pm$ 3.6%*	-42.5 $\pm$ 2.4%*

Results are expressed as % change relative to concentrations measured in control experiments. Each value represents the average  $\pm$  SD ( $n = 3$ ).

\* $P < 0.05$  as compared with the control cells.

**Figure 2**

Representative immunoblots depicting the expression of the D-serine transporters ASCT2 and Asc1 in immortalized 1321N1 and PC-12 cells, and primary cultures of rat neuronal cells isolated from the cortex (C) and hippocampus (H). C6 glioma cell lysates were used as a positive control. The molecular mass of each transporter (in kDa) is indicated on the right.

well as ASCT1, in each of the cell types studied. However, the specific interaction between ASCT1 and (*S*)-ketamine and (*R*)-ketamine was not pursued because of previous reports indicating that Asc-1 and ASCT2 are primarily responsible for D-serine transport in retinal Müller cells, neuronal cells and astrocytes (Dun *et al.*, 2007; Rosenberg *et al.*, 2013; Martineau *et al.*, 2014).

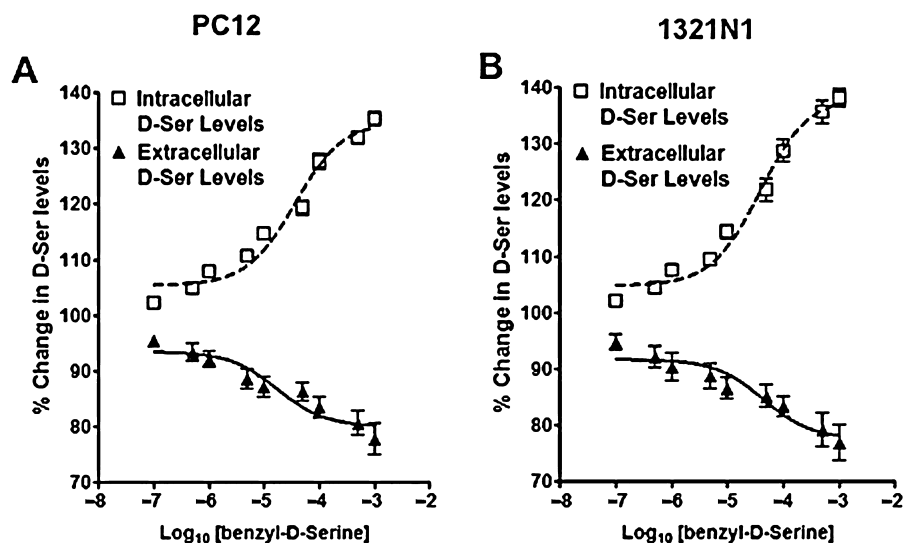
To assess the contribution of ASCT2 to the control of D-serine transport, PC-12, 1321N1, cortex-derived and hippocampus-derived cells were incubated with BDS, a selective ASCT2 competitive inhibitor (Greuer and Grabsch, 2004). Treatment of PC-12 and 1321N1 cells with BDS (0–1000  $\mu$ M) produced significant concentration-dependent increases in the intracellular D-serine levels, with maximum increases of 35.3  $\pm$  3.8 and 38.2  $\pm$  8.0% and corresponding EC<sub>50</sub> values of 53.9  $\pm$  6.5 and 38.1  $\pm$  4.7  $\mu$ M, respectively (Figure 3A,B). There were corresponding decreases in the extracellular D-serine concentrations, with maximum

decreases of 25.2  $\pm$  9.8% (PC-12) and 23.2  $\pm$  9.5% (1321N1) and IC<sub>50</sub> values of 64.9  $\pm$  18.3 and 52.5  $\pm$  13.2  $\mu$ M, respectively (Figure 3A,B).

The incubation of PC-12 cells with 0.5  $\mu$ M (*S*)-ketamine (the approximate EC<sub>50</sub> – IC<sub>50</sub> value) produced a significant 26.1  $\pm$  1.9% increase in the intracellular D-serine levels and a 17.9  $\pm$  1.9% decrease in the extracellular D-serine concentration (see Figure 1A,B). The incubation of PC-12 cells with 50  $\mu$ M BDS (the approximate EC<sub>50</sub> – IC<sub>50</sub> value) produced a 27.4  $\pm$  5.1% increase in the amount of intracellular D-serine and a 14.8  $\pm$  3.8% decrease in the extracellular D-serine levels (see Figure 3A). Co-incubation with (*S*)-ketamine (0.5  $\mu$ M) and BDS (50  $\mu$ M) resulted in a 47.8  $\pm$  0.9% increase in the intracellular D-serine concentration and a 34.5  $\pm$  3.7% decrease in the extracellular D-serine concentration (data not shown), indicating that the effects of (*S*)-ketamine and BDS were additive.

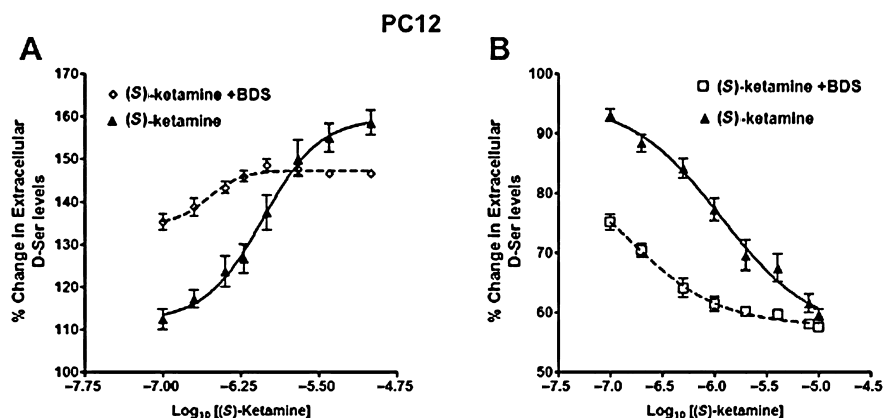
The incubation of primary cortical and hippocampal neuronal cells with BDS (50  $\mu$ M) produced the same qualitative and significant changes in the intracellular and extracellular concentrations of D-serine as observed in the immortalized cell lines. BDS increased the amount of intracellular D-serine by 21.6  $\pm$  1.9% in the cortex-derived cells and 22.2  $\pm$  1.9% in the hippocampus-derived cells, and decreased the extracellular D-serine levels by 18.5  $\pm$  2.8 and 16.5  $\pm$  1.6%, respectively (Table 1). Co-incubation with (*S*)-ketamine (0.5  $\mu$ M) and BDS (50  $\mu$ M) resulted in 47.2  $\pm$  5.1% (cortex) and 45.3  $\pm$  3.4% (hippocampus) increases in the intracellular D-serine levels, and corresponding 44.1  $\pm$  3.6 and 42.5  $\pm$  2.4% decreases in the extracellular D-serine concentrations (Table 1), indicating that the effects of (*S*)-ketamine and BDS were also additive in these cells.

The interaction between (*S*)-ketamine and BDS was further investigated in PC-12 cells by the co-incubation of BDS (50  $\mu$ M) with (*S*)-ketamine concentrations ranging from 0.1 to 10  $\mu$ M. The resulting effect on the intracellular and extracellular D-serine levels was then determined (Figure 4A,B). The presence of BDS in the incubation media shifted the concentration–response curves produced by



**Figure 3**

Effect of benzyl-D-serine (BDS) on the cellular partitioning of D-serine in PC-12 and 1321N1 cells. PC-12 (A) and 1321N1 cells (B) were incubated with increasing concentrations of the ASCT2 inhibitor BDS (0–1000  $\mu\text{M}$ ) for 36 h, followed by the determination of the intracellular and extracellular D-serine contents. The  $\text{EC}_{50}$  and  $\text{IC}_{50}$  values were calculated and presented in the Results section. The  $\text{EC}_{50}$  and  $\text{IC}_{50}$  values for BDS in PC-12 cells, based upon the extracellular and intracellular D-Ser levels, were  $64.95 \pm 1.83$  and  $53.92 \pm 6.53$   $\mu\text{M}$ , whereas in 1321N1 cells, the  $\text{IC}_{50}$  values were  $38.16 \pm 8.01$  and  $52.50 \pm 13.12$   $\mu\text{M}$ . Data represent the average  $\pm$  SD of three independent experiments.



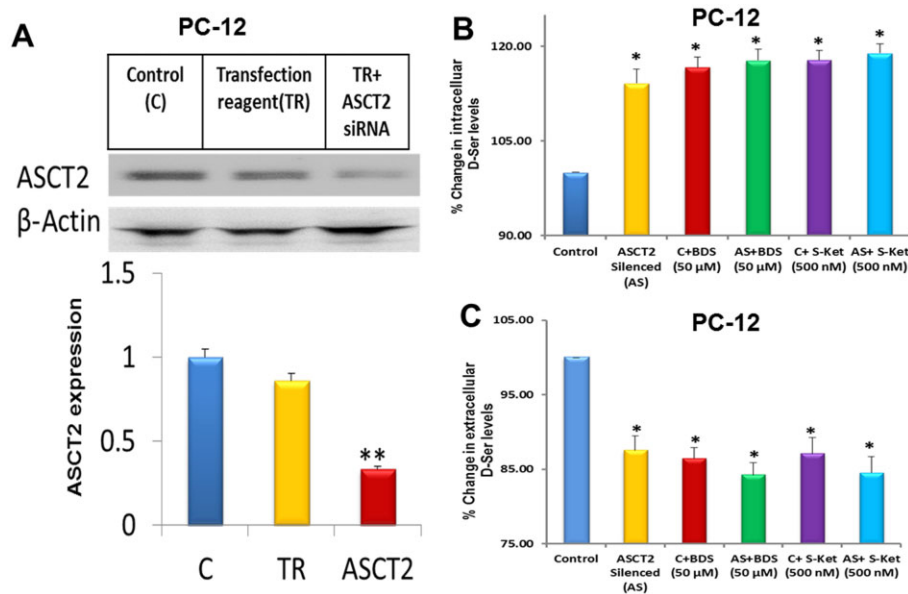
**Figure 4**

Interaction between (*S*)-ketamine and benzyl-D-serine (BDS) in PC-12 cells. Cells were incubated with increasing concentrations of (*S*)-ketamine (0–10  $\mu\text{M}$ ) in the presence or absence of BDS (50  $\mu\text{M}$ ) for 36 h followed by the determination of intracellular (panel A) and extracellular (panel B) D-serine content. Data represent the average  $\pm$  SD of three independent experiments.

(*S*)-ketamine to the left and resulted in approximately three-fold reductions in the  $\text{EC}_{50}$  and  $\text{IC}_{50}$  values to  $0.28 \pm 0.02$  and  $0.27 \pm 0.01$   $\mu\text{M}$  respectively.

In the next series of experiments, ASCT2 gene knockdown was accomplished in PC-12 cells using a pool of siRNA. An initial experiment established that the expression of ASCT2 protein was significantly reduced ( $P < 0.01$ ) after 24 h of siRNA treatment without an effect on  $\beta$ -actin expression (Figure 5A). Reducing the expression of ASCT2 produced a

significant  $14.1 \pm 2.2\%$  increase in the intracellular D-serine concentrations (Figure 5B) and lower extracellular D-serine levels ( $12.4 \pm 1.9\%$ ; Figure 5C), while cell treatment with the transfection reagent alone had no effect (data not shown). Incubation with BDS (50  $\mu\text{M}$ ) and (*S*)-ketamine (0.50  $\mu\text{M}$ ) produced the same significant increases in the intracellular D-serine content and corresponding decreases in the extracellular concentration of D-serine as those observed in the siRNA-treated cells and without additive effects (Figure 5B,C).



**Figure 5**

Effect of ASCT2 silencing on the cellular response to benzyl-D-serine (BDS) and (*S*)-ketamine. (A) Top panel: Representative autoradiogram depicting the expression of ASCT2 and  $\beta$ -actin proteins after siRNA-mediated ASCT2 knockdown in PC-12 cells. Bottom panel: bars represent the relative expression levels of ASCT2 after normalization with  $\beta$ -actin. (B, C) Effect of BDS (50  $\mu$ M) and (*S*)-ketamine (250 nM) on the intracellular (panel B) and extracellular (panel C) D-serine levels in both control, untransfected PC-12 cells (C), cells incubated with transfection reagent alone (TR), and ASCT2-silenced PC-12 cells (AS). Data represent the average  $\pm$  SD of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$  as compared with the control cells.

Thus, ASCT2 silencing conferred refractoriness to BDS and (*S*)-ketamine signalling with regard to the cellular accumulation and export of D-serine.

The contribution of the Asc-1 transporter to the observed effects produced by (*S*)-ketamine and BDS was investigated using D-isoleucine, a selective agonist of Asc-1 antiporter activity (Rosenberg *et al.*, 2013). Incubation of PC-12 cells with increasing concentrations of D-isoleucine (0–2000  $\mu$ M) produced a concentration-dependent increase in the intracellular D-serine levels with a calculated  $EC_{50}$  value of  $197.20 \pm 6.84$   $\mu$ M (Figure 6A). There were corresponding decreases in the amount of extracellular D-serine with an  $IC_{50}$  value of  $179.6 \pm 9.88$   $\mu$ M (Figure 6B). Incubation of PC-12 cells with 200  $\mu$ M D-isoleucine (the  $\sim EC_{50}/IC_{50}$  concentration) decreased the intracellular D-serine concentration by  $19 \pm 2\%$ , while incubation with 0.6  $\mu$ M D-isoleucine (the  $\sim EC_{50}/IC_{50}$  concentration) increased the intracellular D-serine levels by  $22 \pm 4\%$  (Table 2). Co-incubation with D-isoleucine (200  $\mu$ M) and (*S*)-ketamine (0.6  $\mu$ M) produced a  $16 \pm 2\%$  decrease in intracellular D-serine, which was slightly lower, albeit significant, relative to the decrease produced by D-isoleucine alone. Incubation of PC-12 cells with 200  $\mu$ M D-isoleucine increased the extracellular D-serine concentration by  $21 \pm 2\%$ , while incubation with 0.6  $\mu$ M (*S*)-ketamine decreased the amount of extracellular D-serine by  $20 \pm 4\%$  (Table 2). Co-incubation with D-isoleucine (200  $\mu$ M) and (*S*)-ketamine (0.6  $\mu$ M) produced a  $17 \pm 1\%$  increase, which was slightly lower, albeit significant, than the effect produced by D-isoleucine alone.

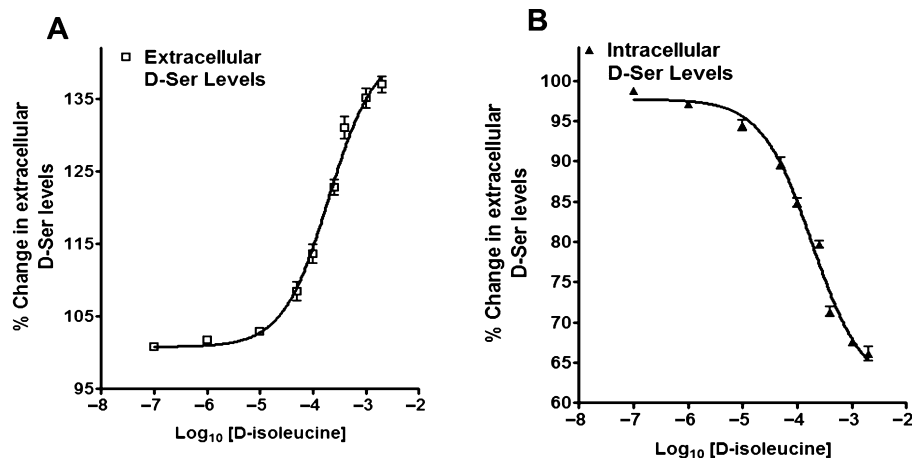
Western blotting analysis established that (*S*)-ketamine and (*R*)-ketamine produced significant increases in the mono-

meric form of serine racemase (m-SR) protein in PC-12 and 1321N1 cells (Figure 7). In PC-12 cells, the maximum increase ( $\sim 2.0$ -fold) in m-SR expression was observed at 4 and 0.2  $\mu$ M for (*R*)-ketamine and (*S*)-ketamine, respectively (Figure 7A,B). Similarly, the maximum effects on m-SR expression were observed at 2  $\mu$ M for (*R*)-ketamine and 0.5  $\mu$ M for (*S*)-ketamine in 1321N1 cells (Figure 7C,D). The data indicate that (*S*)-ketamine and (*R*)-ketamine produced U-shaped concentration-dependent responses on m-SR expression and that the effect was enantiospecific, with (*S*)-ketamine being the more potent enantiomer. Treatment with BDS or D-isoleucine and the siRNA-mediated attenuation of ASCT2 expression had no effect on m-SR expression in these two immortalized cell lines (data not shown). Incubation of cortex-derived cells and hippocampus-derived cells with (*R*)-ketamine (1  $\mu$ M) increased the expression of m-SR by  $\sim 1.5$ -fold, while an  $\sim 2.0$ -fold increase was observed with (*S*)-ketamine (0.5  $\mu$ M) (Figure 7E,F). The expression of d-SR was not affected by any of the treatments used in this study (data not shown).

## Discussion and conclusions

The anaesthetic and antinociceptive properties of (*R,S*)-ketamine have been associated with direct non-competitive inhibition of the NMDA receptor through binding at the receptor's PCP-binding site (Kohrs and Durieux, 1998; Hirota and Lambert, 2011). (*S*)-ketamine displays an approximately threefold higher affinity for the NMDA receptor relative to





**Figure 6**

Effect of D-isoleucine on the cellular partitioning of D-serine in PC-12 cells. Cells were incubated with increasing concentrations of D-isoleucine (0–2000  $\mu$ M) for 36 h followed by the determination of (A) extracellular and (B) intracellular D-serine levels. Data represent the average  $\pm$  SD of three independent experiments.

**Table 2**

The change in intracellular and extracellular D-serine concentrations in PC-12 cells produced by incubation with D-isoleucine and (S)-ketamine

Samples	% change in intracellular D-serine levels	% change in extracellular D-serine levels
Control	100.00	100.00
D-IL (200 $\mu$ M)	$-19.2 \pm 1.8\%^*$	$+21.4 \pm 1.7\%^*$
S-Ket (0.5 $\mu$ M)	$+22.0 \pm 3.5\%^*$	$-20.2 \pm 3.6\%^*$
D-IL + S-Ket	$-15.7 \pm 1.9\%^*$	$+16.6 \pm 0.7\%^*$

The results are expressed as % change relative to concentrations measured in control experiments. Each value represents the average  $\pm$  SD ( $n = 3$ ).

\* $P < 0.05$  as compared with the control cells.

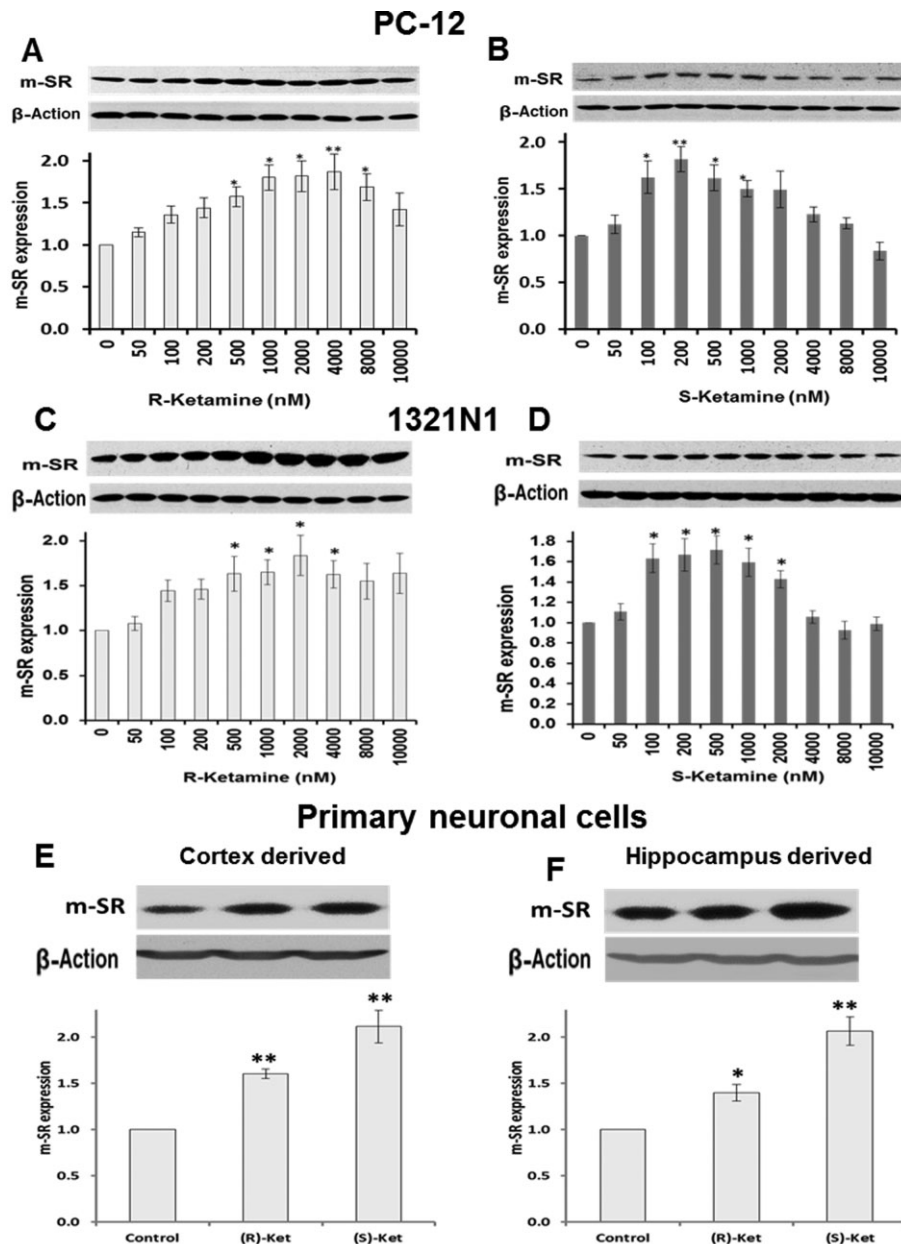
(R)-ketamine, and this variance occurs in conjunction with clinical differences between the two enantiomers, including the dissociative effects associated with the sub-anaesthetic dose of the drug (Vollenweider *et al.*, 1997; Persson *et al.*, 2002; Domino, 2010). In particular, studies in healthy volunteers indicate that (S)-ketamine produces more profound dissociative effects, ego-disintegration and hallucinatory phenomena than (R)-ketamine (Vollenweider *et al.*, 1997; Persson *et al.*, 2002).

Although the direct inhibition of NMDA receptor activity and the resulting NMDA receptor hypofunction, the ‘hypoglutamatergic hypothesis’, is the accepted explanation of the psychosis-inducing effect of (R,S)-ketamine in healthy volunteers (Pomarol-Clotet *et al.*, 2006), an indirect attenuation of NMDA receptor function via the down-modulation of D-serine plasma concentrations could also explain the increase in CADSS scores (Moaddel *et al.*, 2015), as illustrated in Figure 8A. This hypothesis is based upon the function of

D-serine as a key and potent NMDA receptor co-agonist (Wolosker *et al.*, 2008). Synaptic NMDA receptors have a preferential affinity for D-serine relative to the NMDA receptor co-agonist glycine, and D-serine plays a key role in LTP and NMDA-induced neurotoxicity (Henneberger *et al.*, 2010; 2013; Papouin *et al.*, 2012). In addition, subjects with schizophrenia have significantly lower baseline D-serine plasma concentrations relative to healthy controls and there is a negative association of D-serine plasma levels with severity of the symptoms of the disease (Hashimoto *et al.*, 2003; Calcia *et al.*, 2012).

The effect of D-serine on LTP has been linked to its release from astrocytes (Henneberger *et al.*, 2010; Kang *et al.*, 2013), and D-serine release from primary neuronal cultures obtained from rat cortex and hippocampus has been associated with NMDA receptor activation (Kartvelishvily *et al.*, 2006). D-Serine release is mediated by ASCT2 and Asc-1 (Sikka *et al.*, 2010; Maucler *et al.*, 2013; Rosenberg *et al.*, 2013; Martineau *et al.*, 2014). In particular, the Asc-1-mediated release of neuronal D-serine has been shown to play a key role in LTP in rat and mouse models (Rosenberg *et al.*, 2013), while ASCT2 plays an important role in D-serine release from astrocytes and postsynaptic neurons (Martineau *et al.*, 2014).

In the current study, we have investigated the effect of (S)-ketamine and (R)-ketamine on ASCT2- and Asc-1-mediated cellular export of D-serine in PC-12 and 1321N1 cells and in primary neuronal cultures obtained from rat cortex and hippocampus. The immortalized cell lines and primary neuronal cells expressed Asc-1 and ASCT2 proteins. Incubation of PC-12 and 1321N1 cells with (R)-ketamine produced a concentration-dependent decrease in the intracellular and extracellular D-serine levels. Moreover, incubation of the cortex-derived and hippocampus-derived primary neuronal cells with (R)-ketamine (1.0  $\mu$ M) also decreased the amount of intracellular and extracellular D-serine. The observed reductions were consistent with previous studies showing that the diminution in the intracellular D-serine concentrations was associated with the inhibition of the *de*

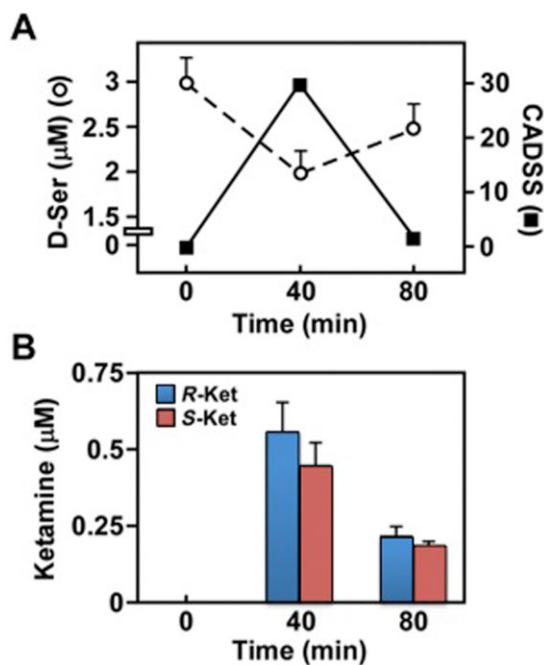


**Figure 7**

Effect of (*R*)-ketamine and (*S*)-ketamine on the expression of monomeric serine racemase protein. (A–D) Thirty-six hours after treatment with the indicated concentrations of (*R*)-ketamine (panels A and C) or (*S*)-ketamine (panels B and D), PC-12 (panels A and B) and 1321N1 (panels C and D) cell lysates were prepared and then immunoblotted with anti-serine racemase (m-SR) antibody. Relative levels of m-SR after quantification and normalization with  $\beta$ -actin are shown in the bars. (E, F), Primary cultures of rat neuronal cells isolated from cortex (panel E) and hippocampus (panel F) were treated with vehicle, (*R*)-ketamine (1  $\mu$ M) or (*S*)-ketamine (0.5  $\mu$ M) for 36 h and then processed for m-SR immunoblot analysis. Data represent the average  $\pm$  SD of three independent experiments. \* $P$  < 0.05; \*\* $P$  < 0.01 as compared with the control cells.

*novo* synthesis of D-serine by the ketamine metabolites (*R,S*)-norketamine, (*R,S*)-dehydronorketamine and (*2S,6S*)-hydroxynorketamine (Singh *et al.*, 2013; Paul *et al.*, 2014). This effect was attributed to the non-competitive inhibition of the  $\alpha 7$  and  $\alpha 3\beta 4$  subtypes of the nicotinic acetylcholine (nACh) receptor. These nACh receptor subtypes are expressed in PC-12 and 1321N1 cells (Singh *et al.*, 2013) and Western blot analysis of the cortex-derived and hippocampus-derived

neuronal cells used in this study confirmed the presence of the  $\alpha 7$  and  $\alpha 3$  nACh receptor subunits (data not shown). (*R,S*)-ketamine has been characterized as a non-competitive inhibitor of the  $\alpha 7$  nACh receptor (Coates and Flood, 2001) and  $\alpha 3\beta 4$  subtypes (Moaddel *et al.*, 2013), and (*R*)-ketamine and (*S*)-ketamine have been identified as non-competitive inhibitors in PC-12 cells (Sasaki *et al.*, 2000) and human neuroblastoma SH-SY5Y cells (Friederich *et al.*, 2000); the latter



**Figure 8**

Correlation between CADSS score, plasma D-serine concentration and (*R*)- and (*S*)-ketamine levels in MDD patients, based upon the data reported by Moaddel *et al.* (2015). (A) Left Y-axis: The effect of a 40 min i.v. infusion of 0.5 mg·kg<sup>-1</sup> (*R,S*)-ketamine on the plasma concentration of D-serine in MDD patients was determined from baseline post-infusion levels. Right Y-axis: Changes in the average CADSS scores over time in MDD patients following administration of (*R,S*)-ketamine. (B) The plasma concentrations of (*R*)-ketamine and (*S*)-ketamine following a 40 min i.v. infusion of 0.5 mg·kg<sup>-1</sup> (*R,S*)-ketamine in MDD patients were determined from baseline post-infusion levels.

cell type also expressing  $\alpha 7$  and  $\alpha 3\beta 4$  nACh receptors (Dajas-Ballador *et al.*, 2002; Dunckley and Lukas, 2006). Thus, the inhibition of D-serine synthesis by (*R*)-ketamine in the immortalized and primary cells was expected and consistent with previous data.

Since (*S*)-ketamine is also a non-competitive nACh receptor inhibitor, it was assumed that (*S*)-ketamine would also produce a concentration-dependent decrease in the intracellular D-serine concentrations. Unexpectedly, (*S*)-ketamine induced a concentration-dependent increase in the intracellular D-serine levels both in immortalized PC-12 and 1321N1 cells and following incubation of the cortex-derived and hippocampus-derived primary neuronal cells with (*S*)-ketamine (0.5  $\mu$ M). The corresponding decrease in the extracellular D-serine levels suggested that the intracellular and extracellular changes in D-serine might be associated with the inhibition of the active export of the compound by Asc-1 and/or ASCT2. This mechanism was investigated using the specific ASCT2 inhibitor BDS (Greuer and Grabsch, 2004) in all of the experimental cells and ASCT2 gene knockdown in PC-12 cells. Both approaches produced the same change in the intracellular/extracellular D-serine distribution in response to (*S*)-ketamine. The results establish that (*S*)-

ketamine reduces D-serine transport through ASCT2 inhibition, while (*R*)-ketamine has no effect.

The action of (*S*)-ketamine on ASCT2 transport was further examined by co-incubation of PC-12 cells with (*S*)-ketamine and BDS using the approximate EC<sub>50</sub> – IC<sub>50</sub> values of both compounds. An apparent additive increase in the amount of intracellular D-serine with a corresponding reduction in the extracellular concentrations of D-serine was observed. The incubation of primary cortical and hippocampal neuronal cells with either BDS or (*S*)-ketamine alone and in combination produced the same qualitative and significant changes in the intracellular and extracellular levels of D-serine as observed in the immortalized cell lines. The data suggest that both compounds are competitive inhibitors of ASCT2 vis-à-vis D-serine transport. The interaction between (*S*)-ketamine and BDS was further investigated in PC-12 cells by the co-incubation of BDS (50  $\mu$ M) with (*S*)-ketamine concentrations ranging from 0.1 to 10  $\mu$ M. The presence of BDS in the incubation media shifted the concentration–response curves produced by (*S*)-ketamine to the left and resulted in approximately threefold reductions in the EC<sub>50</sub> (increase in the intracellular D-serine concentrations) and IC<sub>50</sub> values (decrease in the extracellular D-serine levels). The results of these studies indicate that the inhibition of ASCT2 transport of D-serine by (*S*)-ketamine is multimodal with both competitive and high affinity non-competitive inhibition of ASCT2.

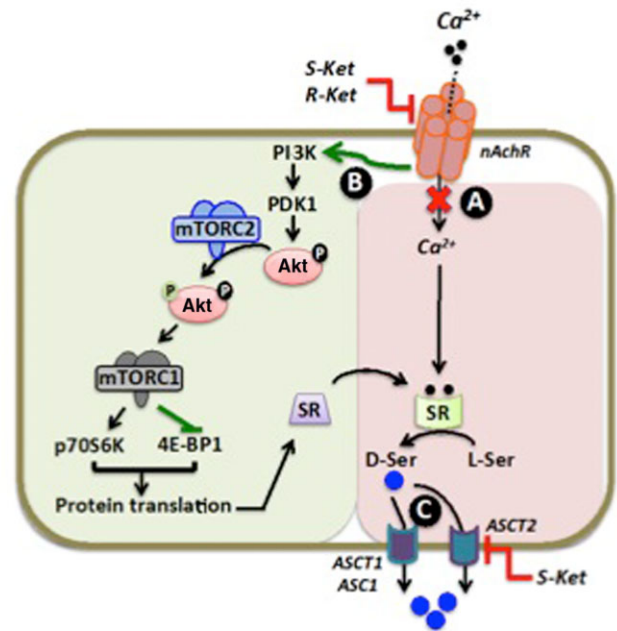
The data from this study also demonstrate that incubation with (*R*)-ketamine and (*S*)-ketamine resulted in a significant increase in the m-SR expression with an inverted U-shaped dose–response curve in all the experimental cell types. (*S*)-ketamine was ~10-fold more potent than (*R*)-ketamine in PC-12 and 1321N1 cells, and similar enantioselectivity was observed in the cortex-derived and hippocampus-derived primary neuronal cells as incubation with (*S*)-ketamine (0.5  $\mu$ M) produced a significantly greater increase in the expression of m-SR than (*R*)-ketamine (1.0  $\mu$ M). The results are consistent with our previous findings, which showed that the incubation of PC-12 cells with (*R,S*)-ketamine concentrations increased the m-SR expression via activation of the mammalian target of rapamycin (mTOR) pathway (Paul *et al.*, 2014). The increase in *de novo* protein synthesis was initiated by non-competitive allosteric inhibition of the  $\alpha 7$ -nACh receptor (Singh *et al.*, 2013; Paul *et al.*, 2014), a process that was blocked by co-incubation with (*S*)-nicotine (Paul *et al.*, 2014). The data presented herein suggest that the antagonistic effect of ketamine at nACh receptors is enantioselective, with (*S*)-ketamine being the more potent inhibitor. Earlier reports have demonstrated that (*S*)-ketamine is an approximately fourfold more potent inhibitor of nACh receptor activity than (*R*)-ketamine in human SH-SY5Y neuroblastoma cells (Friederich *et al.*, 2000), while Sasaki *et al.* (2000) found no significant difference between ketamine enantiomers in PC-12 cells. Both of these studies were conducted as part of the investigations into the anaesthetic effect of ketamine and may have missed enantioselective differences at the lower drug concentrations used in antidepressant therapy.

The modulation in the m-SR expression by both (*S*)-ketamine and (*R*)-ketamine indicates that these isomers should produce similar reductions in the intracellular and extracellular D-serine concentrations through the inhibition of nACh receptors. This is difficult to observe even though

dramatic and opposite concentration-dependent changes in the intracellular D-serine concentrations were noted in PC-12 and 1321N1 cells. However, the enantioselective effect on the extracellular D-serine levels is more subtle and quantitative. While both (S)-ketamine and (R)-ketamine had a significantly different effect on the extracellular D-serine concentrations, these effects did not reach significance in the PC-12 cells until a 2.0  $\mu\text{M}$  concentration of (S)-ketamine and (R)-ketamine, and, in 1321N1 cells, a concentration of 4.0  $\mu\text{M}$  was required to produce a significant difference between the enantiomers (Figure 1B,D). These results suggest that the effect of (S)-ketamine on the amount of extracellular D-serine is due to both the reduction in intracellular synthesis and the inhibition of active export.

Previous studies have determined that D-serine release from primary neuronal cultures and immortalized cell lines is primarily mediated by Asc-1 (Kartvelishvily *et al.*, 2006; Sikka *et al.*, 2010; Maucler *et al.*, 2013; Rosenberg *et al.*, 2013; Martineau *et al.*, 2014). D-isoleucine is an Asc-1 agonist that increases cellular export of D-serine (Rosenberg *et al.*, 2013). As expected, incubation of PC-12 cells with D-isoleucine led to a significant decrease in intracellular D-serine and a corresponding increase in the extracellular D-serine levels. Co-incubation of PC-12 cells with D-isoleucine (200  $\mu\text{M}$ ) and (S)-ketamine (0.6  $\mu\text{M}$ ), the approximate  $\text{IC}_{50}/\text{EC}_{50}$  concentrations of the two agents, produced a slight, but significant, attenuation of the D-isoleucine response; moreover, increasing the concentration of (S)-ketamine to 10  $\mu\text{M}$  did not alter D-isoleucine responsiveness (data not shown). The results suggest that (S)-ketamine does not directly compete with D-isoleucine and that the observed reduction in D-isoleucine actions stemmed from the pharmacological inhibition of ASCT2 by (S)-ketamine. Similar results were observed when BDS was used as co-incubate (data not shown).

The ~20–25% decrease in D-serine plasma levels observed at the end of the (R,S)-ketamine infusion in MDD patients appears to be clinically relevant and is associated with a corresponding increase in the CADSS scores of these patients (Moaddel *et al.*, 2015) (Figure 8A). Moreover, the rapid fall in the CADSS scores between the 40 and 80 min sampling points was associated with the rapid plasma clearance of (S)-ketamine (an ~50% drop during that time period) (Moaddel *et al.*, 2015) (Figure 8B), suggesting the contribution of (S)-ketamine in this effect. (R,S)-ketamine is extensively metabolized by microsomal enzymes with a major metabolic pathway involving N-demethylation to norketamine and further transformation to (R,S)-dehydronorketamine and a series of diastereomeric hydroxynorketamines (Kharasch and Labroo, 1992; Portmann *et al.*, 2010; Desta *et al.*, 2012). It is possible that one or more of these metabolites also contribute to the rapid drop in D-serine plasma concentrations. A recent study examined the contribution of (S)-norketamine to (S)-ketamine-induced acute pain relief and neurocognitive impairment in healthy volunteers and concluded that (S)-norketamine made no contribution to the cognitive impairment produced by the administration of (S)-ketamine (Olofsen *et al.*, 2012). We have also demonstrated that (R,S)-dehydronorketamine reduces the intracellular D-serine concentrations (Singh *et al.*, 2013) and preliminary data from recent studies indicate that the individual stereoisomers of norketamine, dehydronorketamine and hydroxynorketamine



**Figure 9**

Schematic representation of the regulation of endogenous D-serine level. (A) Inhibition of nACh receptors by (R)-ketamine and (S)-ketamine attenuates the entry of extracellular  $\text{Ca}^{2+}$ . (B) Activation of the PI3K/Akt/mTOR pathway increases serine racemase (SR) expression; however, SR activity is reduced due to a decrease in intracellular  $\text{Ca}^{2+}$ . (C) The contribution of the neutral amino acid transporters, ASCT2, ASCT1 and Asc1, to the export of D-serine and the enantioselective inhibition of ASCT2 by (S)-ketamine is also depicted.

attenuate the intracellular D-serine concentrations and, like (R)-ketamine, have no effect on D-serine transport by ASCT2 (unpublished data). In addition, previous studies in MDD patients indicate that D-serine plasma levels return to the approximate pre-dose level by 80–120 min post-dosing and then slowly decrease over a 7 day period to an average of 39% decrease in patients who respond to (R,S)-ketamine treatment and 28% in patients who do not respond (Moaddel *et al.*, 2015). Thus, it appears that D-serine plasma concentrations are affected by two independent mechanisms, an immediate and steep decrease associated with (S)-ketamine inhibition of ASCT2-mediated transport and a longer, gradual decrease caused by ketamine and ketamine metabolite inhibition of nACh receptors and the resulting decrease in SR activity.

The data from this study expand our understanding of the clinically relevant mechanisms associated with the use of (R,S)-ketamine in the treatment of depression. The additional insight is related to the dissociative effect of the drug through the selective inhibition of ASCT2 by (S)-ketamine, as illustrated in Figure 9. This property of (S)-ketamine may be associated with the increase in the cerebral metabolic rates of glucose in the frontal cortex and ego-disintegration and hallucinatory phenomena produced by the drug. In contrast, the lack of ASCT2 inhibitory activity by (R)-ketamine may be reflected in the development of a state of relaxation (Vollenweider *et al.*, 1997). A recent report has suggested that

(R)-ketamine may be a better antidepressant than (S)-ketamine (Zhang *et al.*, 2014). Our study did not investigate the relative antidepressant efficacy of (S)-ketamine and (R)-ketamine and, therefore, the data provide no insight into the overall clinical response. However, the results indicate that the treatment-associated dissociative effects observed with the administration of (R,S)-ketamine might be reduced by utilization of the (R)-ketamine alone and provide a mechanistic basis for this hypothesis.

## Acknowledgements

This work was supported by funding from the Intramural Research Program of the National Institute on Aging/NIH and by NIA Contract No. HHSN2712010000081.

## Author contributions

N. S. S. did the experimental design and execution, interpretation of data and manuscript preparation. M. B. contributed conceptually and assisted in manuscript preparation. M. A. K. and R. M. provided technical support. S. C. and M. P. M. provided primary culture of rat neuronal cells. I. W. W. contributed conceptually, did the interpretation of data, manuscript preparation and was the project leader.

## Conflict of interest

The authors declare the absence of conflict of interest.

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