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ORIGINAL ARTICLE

Argon blocks the expression of locomotor sensitization to amphetamine through antagonism at the vesicular monoamine transporter-2 and mu-opioid receptor in the nucleus accumbens

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We investigated the effects of the noble gas argon on the expression of locomotor sensitization to amphetamine and amphetamine-induced changes in dopamine release and mu-opioid neurotransmission in the nucleus accumbens. We found (1) argon blocked the increase in carrier-mediated dopamine release induced by amphetamine in brain slices, but, in contrast, potentiated the decrease in KCl-evoked dopamine release induced by amphetamine, thereby suggesting that argon inhibited the vesicular monoamine transporter-2; (2) argon blocked the expression of locomotor and mu-opioid neurotransmission sensitization induced by repeated amphetamine administration in a short-term model of sensitization in rats; (3) argon decreased the maximal number of binding sites and increased the dissociation constant of mu-receptors in membrane preparations, thereby indicating that argon is a mu-receptor antagonist; (4) argon blocked the expression of locomotor sensitization and context-dependent locomotor activity induced by repeated administration of amphetamine in a long-term model of sensitization. Taken together, these data indicate that argon could be of potential interest for treating drug addiction and dependence.

INTRODUCTION

Repeated exposure to amphetamine and amphetamine-derived drugs is well known to produce behavioral changes. This includes locomotor sensitization, which is characterized by an enhanced locomotor response to a subsequent psychostimulant challenge. The effects of the psychostimulant drugs that belong to the amphetamine family are thought to result from an increase in dopamine release in limbic brain regions,[1–3] particularly the nucleus accumbens whose critical role in behavioral sensitization to amphetamine is well established.[4,5] However, apart from the dopaminergic neurotransmission, other neurotransmitter systems, such as the mu-opioid neurotransmission,[6] are thought to contribute directly, or indirectly through interactions with the dopaminergic neurotransmission, to the effects of amphetamine and amphetamine-derived drugs.

Parallel to these studies, a series of *in vitro* and *in vivo* studies has clearly demonstrated the potentially therapeutic properties of the inert gases xenon, nitrous oxide and argon.[7–20] Particularly, in line with their antagonistic action at the *N*-methyl-D-aspartate glutamate receptor and nicotinic acetylcholine receptor,[21–26] xenon and nitrous oxide at subanesthetic concentrations have been further shown to block the development of locomotor sensitization to amphetamine,[27] but not, so far as nitrous oxide is concerned, its expression (personal data). In contrast with xenon and nitrous oxide, the non-anesthetic gas argon has been shown

to produce narcosis at hyperbaric pressure through activation of the GABA type A (GABA-A) and benzodiazepine receptors.[28] Accordingly, in line with previous studies that have reported that prototypical GABA-A and benzodiazepine receptor agonists blocked the acquisition of locomotor sensitization to amphetamine-derived drugs,[29,30] argon has been further shown to prevent the acquisition of locomotor sensitization to amphetamine and the concomitant increase in accumbal mu-receptor activity induced by repeated administration of amphetamine.[31] But, whether argon could block the expression of locomotor sensitization to amphetamine—a condition that we believe to be prerequisite for evaluating actually the real potential of argon as a possible therapeutic agent for the treatment of drug addiction—still remains to be investigated.

The present study was designed to investigate the effects of argon on the expression of neurobehavioral sensitization to amphetamine. We first investigated the effects of argon on locomotor sensitization and amphetamine-induced changes in dopaminergic and mu-opioidergic neurotransmission in the nucleus accumbens in a short-term model of sensitization and withdrawal to amphetamine. Given the results obtained, we further investigated the effects of argon on locomotor sensitization and context-dependent locomotor activity induced by amphetamine in a long-term, more clinically relevant, model of sensitization and withdrawal to amphetamine.

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Received 29 September 2014; revised 6 January 2015; accepted 20 January 2015
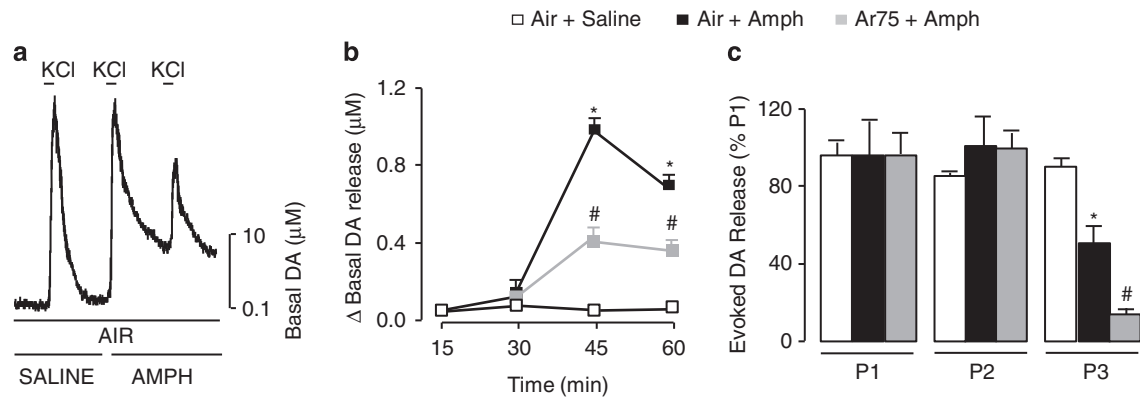
Figure 1. Effects of argon on amphetamine-induced changes in carrier-mediated- and KCl-evoked dopamine release in brain slices. (**a**) Representative experimental profile showing the effects of amphetamine (AMPH) on carrier-mediated- and KCl-evoked dopamine release. Amphetamine caused an increase in carrier-mediated dopamine release and a concomitant reduction of Peak 3 (P3), but not of Peak 2 (P2), KCl-evoked dopamine (DA) release expressed as a percentage of Peak 1 (P1) taken as a 100% value. (**b**) Argon decreased the amphetamine-induced increase in carrier-mediated dopamine release. (**c**) Argon potentiated the decrease in KCl-evoked dopamine release induced by amphetamine. Data are expressed as the mean ± s.e.m.; *n* = 4 per condition. *$P < 0.02$ vs control slices (saline+air); #$P < 0.02$ vs amphetamine +air.

MATERIALS AND METHODS

Animals

All animal-use procedures were in accordance with the Declaration of Helsinki and the framework of the French legislation for the use of animals in biomedical studies. All experiments were approved by a research ethics committee. Male adult Sprague Dawley rats (Janvier, Le Genest Saint-Isle, France) weighing 250–300 g were used. Rats were housed socially in groups of 3 to 4 at 21.5 °C in perspex home cages with free access to food and water. Light was maintained on a reverse light–dark cycle, with lights on from 2000 to 0800 h.

Dopamine-release studies

Preparation and incubation of brain slices. Rats were killed by decapitation. The brains were carefully removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing in mM: 4.9 KCl, 118 NaCl, 1.18 MgCl₂, 1.25 NaH₂PO₄, 1.25 CaCl₂, 3.6 NaHCO₃, 10 d-glucose, 30 HEPES. Coronal brain slices (400 μm thickness) including the nucleus accumbens (anteriority: −1.2 to +2 mm from the bregma) were cut using a tissue chopper (Mickie Laboratory Engineering, Gomshall, UK). Brain slices (*n* = 4 per condition) were transferred to an isolated brain slice chamber containing freshly prepared oxygenated aCSF and allowed to recover at room temperature for at least 1 h. Slices were then placed in a recording chamber (1 ml volume) at 34.5 ± 0.5 °C, and superfused at a flow rate of 1 ml min⁻¹ with aCSF saturated with air (75 vol% nitrogen+25 vol% oxygen). Following a 20-min baseline period, amphetamine (10 mM) was added for 30 min to aCSF in the presence of air or argon at 75 vol% (with the remainder being oxygen). Controls were treated with saline and medicinal air.

Measurement of dopamine release. Carrier-mediated and depolarization-dependent (KCl: 100 mM, 1 min, applied every 15 min) dopamine release in the nucleus accumbens were monitored. This was performed using a Biopulse polarograph (Radiometer, Villeurbanne, France) and standard glass-encased nafion-precoated carbon fiber electrodes 10 μm in diameter and 50 μm long (World Precision Instruments, Aston-Stevenage, Hertfordshire, UK).[27] Briefly, the carbon fiber microelectrode sensitivity for dopamine was improved by applying an electrochemical treatment. This involved placing a carbon fiber microelectrode, a platinum auxiliary electrode and an Ag/AgCl reference electrode in a beaker of phosphate-buffered saline solution (pH = 7.4), and applying a 70-Hz triangular waveform of 0–2.6 V for 20 s vs an Ag/AgCl electrode. After such treatment, dopamine oxidation peak potentials approximately occurred at 100 mV as measured by differential normal pulse voltammetry. Just before each experiment, the oxidation peak potential of the recording carbon fiber microelectrode for dopamine was determined in 10 μM dopamine in aCSF using differential normal pulse voltammetry. The polarograph was then switched from the differential normal pulse voltammetry to the differential pulse amperometry mode, and set at the

dopamine oxidation peak potential, enabling real-time measurements of dopamine release. Signals were fed to a Y-t chart recorder and digitized using an analog to digital converter. The tip of the carbon microelectrode was positioned ~ 70 μm below the brain slice surface in the core of the nucleus accumbens, midway between the lateral ventricle and the anterior commissure. The platinum auxiliary and Ag/AgCl reference electrodes were positioned at a convenient position on the brain slice and used to maintain it in the bath. For each experimental condition (saline+air, amphetamine +air, amphetamine+argon), changes in dopamine release were calculated using each slice as its own control as illustrated in Figure 1a: changes in carrier-mediated dopamine release were calculated as (B2 − B1), (B3 − B1) and (B4 − B1); Peak 2 and Peak 3 KCl-evoked dopamine responses were calculated as a percentage change from Peak 1 KCl-evoked dopamine release taken as a 100% value. Then, between-groups comparisons were performed (see statistical methods). At the end of each experiment, dopamine concentration was quantified by post-experimental calibration of the carbon fiber microelectrode. Neither the amperometric response nor the dopamine oxidation peak potential was altered by the presence of amphetamine, air or argon alone, or in combination in aCSF.

Short-term sensitization studies

Rats (*n* = 7–8 per group) were treated from day 1 to day 3 with either amphetamine (1 mg ml⁻¹ kg⁻¹, intraperitoneally) or saline (1 ml kg⁻¹, intraperitoneally), and then were returned to their home cages immediately. From day 4 to day 6 (that is, during the withdrawal period), rats were treated for 3 h with 'medicinal' air (composed of 75 vol% nitrogen+25 vol% oxygen; control animals) or argon at 75 vol% (with the remainder being oxygen). Both gas mixtures were given at a flow rate of 5 l min⁻¹ in a closed chamber of 100 l volume (65 × 45 × 35 cm), a condition that allowed maintaining carbon dioxide < 0.03 vol% and humidity around 65–70% with the use of soda lime and silica gel, respectively.

Behavioral investigations. On day 7, rats were habituated to the activity boxes for 1 h before being challenged with saline (1 ml kg⁻¹, intraperitoneally) or amphetamine (1 mg ml⁻¹ kg⁻¹, intraperitoneally), and then were recorded for locomotor activity for 1 h 30 min, as detailed previously.[27] Briefly, locomotor activity was quantified using a bank of four individual activity cages measuring 30 × 20 × 20 cm, equipped with horizontal infrared beams, located 3 cm above the floor across the long axis of the cage (Imetronic, Pessac, France). Beam interruptions were detected through an electrical interface and recorded over 10-min intervals on a computer. All the experiments were performed during the animals' dark cycle with the activity boxes kept in the dark.

Binding assays. Mu-receptor activity was assessed in the nucleus accumbens on the basis of the critical roles of the mu-receptor and the nucleus accumbens in behavioral sensitization to amphetamine.[4–6,32] At the end of the amphetamine challenge on day 7, the rats were killed. Their brains were carefully removed, frozen with isopentane and stored at − 20 °C.

For each rat, two coronal sections of 20 μm thickness including the nucleus accumbens (anteriority: +1 mm from the bregma) were cryostat cut at −20 °C, applied to glass slides with a very low nonspecific binding capacity (Superfrost Plus, Menzel-Glaser, Braunscheig, Germany) and stored at −20 °C until required for the binding assays. Saturation binding was performed on rat brain sections as detailed previously.[33] Briefly, brain sections were preincubated twice for 5 min at 4 °C in 50 mM Tris-HCl buffer solution [(hydroxyl-methyl)aminomethane] containing 100 mM NaCl, 1 g l$^{−1}$ bovine serum albumin and 20 mg l$^{−1}$ bacitracin, adjusted to pH 7.4, to dissociate and eliminate potential endogenous ligands. Then, brain sections were incubated for 45 min at 4 °C using 800 μl of buffer solution containing increasing concentrations (0.312, 0.625, 1.25, 2.5, 5 nM) of [^{3}H]DAMGO [(D-ala^2,N-methyl-phe^4,glycol^5)(tyrosyl-3,5-^{3}H)enkephalin, 1 Ci l$^{−1}$, specific radioactivity 66 Ci mmol$^{−1}$]. The amount of nonspecific labeling was assessed using adjacent brain sections in the presence of an excess of naloxone at 10 μM. After incubation, brain sections were quickly washed (30 s) with Tris-HCl buffer containing bovine serum albumin (×1) and then with Tris-HCl buffer alone (×3) at 4 °C to eliminate unbound ligand. A final wash was performed at 4 °C with distilled water to remove excess of buffer salts. Then, brain sections were dried overnight at room temperature and stored until counting. Before being used for image acquisition and data analysis, slides containing brain sections were exposed under tritium-sensitive phosphor screens in the dark for 10 days at −20 °C. Images were then captured with a computer-controlled Cyclone phosphorimaging scanner using the OptiQuant acquisition and analysis software (Packard Instrument Company, Meriden, CT, USA). Optical densities expressed as digital light units per mm^2 over [^{3}H] standard spot were measured. Specific binding was determined by subtracting nonspecific binding from total binding. Saturation binding data were fitted according to a one-site binding (hyperbola) model using Graph Pad prism (Graph Pad Prism 4.02; Graph Pad Software, La Jolla, CA, USA). Changes in the maximal number of binding sites (Bmax in fmol mm$^{−3}$) and dissociation constant (Kd in nM) were calculated, and expressed as a percentage from control values. The ratio of Bmax to Kd was calculated and used to estimate the level of constitutive activity of the mu-opioid receptor neurotransmission.

In vitro binding studies

Membranes were prepared from whole brains of untreated rats (n = 4). Briefly, the brains were crushed and homogenized in 50 mM Tris-HCl buffer solution. Brain homogenates were transferred into vials and centrifuged at 45 000 g for 15 min. The bases of the vials were collected and suspended in a same volume of Tris-HCl buffer, incubated and gently agitated for 30 min at 37 °C. Again, the vials were centrifuged, and their bases collected and suspended in Tris-HCl buffer. A solution containing 1 mg proteins per ml was prepared. Saturation binding was performed in Tris-HCl buffer containing bacitracin, bovine serum albumin and [^{3}H]DAMGO at different concentrations (n = 2 per dose) in the presence of naloxone, as described above. Then, the vials were left open, placed in a hyperbaric chamber, and pressurized to and maintained at 0.2 MPa absolute (2 atm) argon (100 vol %) or nitrogen (100 vol%; controls) for 1 h. Then, the vials were decompressed, filtered using a filtermate Perkin-Elmer (Waltham, MA, USA), placed in 24-well plates previously coated with 0.5% polyethyleni-mine and left to dry for one additional hour at 50 °C before being added with 100 μl scintillant to allow radioactivity counting. Specific binding was determined by subtracting nonspecific binding from total binding. Saturation binding data were analyzed as described above. Changes induced by argon in Bmax and Kd values were calculated and expressed as percentage from controls. The ratio of Bmax to Kd was calculated and used to estimate the activity of the mu-opioid neurotransmission in the presence of nitrogen or argon.

Long-term sensitization studies

Three groups of rats (n = 32) were treated with amphetamine from day 1 to day 28 according to an escalating-dose regimen. Each rat was given one daily injection of amphetamine at 2 mg kg$^{−1}$ ml$^{−1}$ from day 1 to day 5, 4 mg kg$^{−1}$ ml$^{−1}$ from day 8 to day 12, 6 mg kg$^{−1}$ ml$^{−1}$ from day 15 to day 19 and 8 mg kg$^{−1}$ ml$^{−1}$ from day 22 to day 26. After each amphetamine injection, the rats were placed immediately in the activity boxes, which were kept in the dark and had wood sawdust on the floor. The rats' locomotor activity was recorded on day 1 for 1 h 30 min to obtain a score of locomotor activity in response to acute amphetamine. On days 6, 7, 13, 14, 20, 21, 27 and 28, the rats were given no injection and allowed to remain in their home cages. From day 29 to day 56, all the rats were given

no injection of amphetamine, but were treated with air and/or argon at 75 vol% for 3 h. The rats of group 1 (control rats, n = 10) and the rats of group 2 (n = 10) were treated, respectively, with air and argon five times a week on days 29–33, 36–40, 43–47 and 50–54. The rats of group 3 (n = 12) were treated once a week with argon on days 29, 36, 43 and 50 and with air four times a week on days 30–33, 37–40, 44–47 and 51–54.

On days 43 and 50, one-half of the animals of each group was placed in activity boxes kept in the dark with sawdust on the floor. Then, they were recorded for context-dependent locomotor activity. The other half of the animals was placed in lightened activity boxes with no sawdust on the floor to assess context-independent locomotor activity. On the day after, that is, on days 44 and 51, the animals that had been recorded for context-dependent locomotor activity were recorded for context-independent locomotor activity, and conversely. On day 58, all the rats were challenged with amphetamine at 2 mg ml$^{−1}$ kg$^{−1}$, and recorded for locomotor activity for 1 h 30 min.

Data presentation and statistical analysis

Data were expressed as the median and quartiles values, and analyzed using nonparametric statistical methods. Following ad hoc analysis of variance, between-group comparisons and within-group comparisons were performed using the Mann–Whitney U-test and Wilcoxon signed-rank t-test, respectively. Statistical significance was set at $P \leqslant 0.05$.

Drugs, chemicals and gases

Amphetamine (d-amphetamine hemisulfate salt, ref. A5880), bovine serum albumin (ref. 2153) and naloxone (naloxone hydrochloride dihydrate, ref. N7758) were purchased from Sigma-Aldrich (Illkirch, France). Bacitracin was purchased from MP biomedicals (Santa Ana, CA, USA), and [^{3}H]DAMGO (66 Ci mmol$^{−1}$) from Amersham Biosciences (Buckinghamshire, UK). Oxygen, nitrogen and argon of medicinal grade were purchased from Air Liquide (Paris, France). Gas mixtures composed of 75 vol% nitrogen+25 vol% oxygen or 75 vol% argon+25 vol% oxygen were obtained using calibrated gas flowmeters and gas analyzers.

RESULTS

Dopamine-release studies

Amphetamine led to a sustained increase in carrier-mediated dopamine release (B3-B4: $U = 0$, $P < 0.02$; Figure 1b) and to a concomitant reduction of Peak 3, but not of Peak 2, KCl-evoked dopamine release ($U = 16$, $P < 0.02$; Figure 1c) as compared with control slices. The lack of effect of amphetamine on Peak 2 KCl-evoked dopamine release doubtlessly resulted from the fact that amphetamine had not yet produced its action.[27]

Argon led to a reduction of the increase in carrier-mediated dopamine release produced by amphetamine. In the presence of argon, carrier-mediated dopamine levels were lower than those recorded in the presence of air and amphetamine (B3-B4: $U = 16$, $P < 0.02$; Figure 1b), but still remained higher than those recorded in control slices (B3-B4: $U = 0.02$, $P < 0.02$; Figure 1b). In contrast with its inhibiting effect on the facilitating action of amphetamine on carrier-mediated dopamine release, argon further dramatically potentiated the reduction in Peak 3 KCl-evoked dopamine release produced by amphetamine in the presence of air ($U = 0$, $P < 0.02$; Figure 1c).

Short-term sensitization studies

Amphetamine-induced changes in locomotor and mu-receptor activity. The effects of amphetamine on locomotor activity and mu-receptor activity are shown in Figure 2. All rats were pretreated with either saline solution or amphetamine, and exposed immediately to medicinal air used as a control gas treatment. When challenged with amphetamine, rats pretreated with repeated administration of saline solution or amphetamine had higher scores of locomotor activity than control rats pretreated and challenged with saline solution ($U = 1$, $P = 0.001$, Figure 2b). Further comparison between rats challenged with amphetamine showed that rats pretreated with repeated
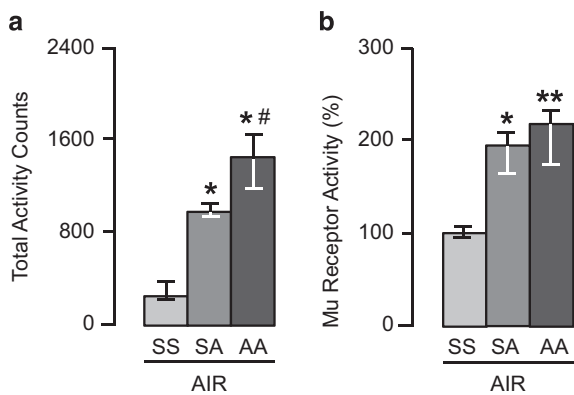
Figure 2. Effects of amphetamine on locomotor activity and mu-receptor neurotransmission in the nucleus accumbens. (**a**) When challenged with amphetamine, rats pretreated with repeated administration of saline solution (SA) or amphetamine (AA) had higher locomotor responses than rats pretreated and challenged with saline solution (SS). Locomotor activity is expressed in arbitrary units. (**b**) As assessed postmortem immediately after being challenged with amphetamine, rats pretreated with repeated administration of saline solution (SA) or amphetamine (AA) had increased mu-receptor activity in the nucleus accumbens (as estimated by the ratio of Bmax to Kd) compared with rats pretreated and challenged with saline solution (SS). Opioid mu-receptor activity in control rats pretreated and challenged with amphetamine was taken as a 100% value. Data are expressed as the median value $\pm$ 25th–75th percentiles; $n = 7$–8 per condition. $*P < 0.005$ and $**P < 0.001$ vs SS; $^{\#}P < 0.001$ vs SA. AA, pretreatment+challenge with amphetamine; SA, pretreatment saline+challenge amphetamine; SS, pretreatment+challenge with saline.

administration of amphetamine had higher scores of locomotor activity than rats pretreated with saline solution ($U = 0$, $P < 0.001$, Figure 2a), thereby indicating that locomotor sensitization to amphetamine had occurred.

As assessed postmortem, immediately after the amphetamine challenge, rats pretreated with repeated administration of saline solution or amphetamine had increased mu-receptor activity (as estimated by the ratio of Bmax to Kd) compared with control rats pretreated and challenged with saline ($U = 4$, $P = 0.005$; $U = 0$, $P < 0.001$; Figure 2b). However, in contrast with what seen for locomotor activity, further comparison between rats challenged with amphetamine revealed no significant difference in mu-receptor activity between rats pretreated with repeated injection of amphetamine and those pretreated with repeated administration of saline solution and air ($U = 32$, NS, Figure 2b).

Effects of argon on amphetamine-induced changes. The effects of argon on locomotor sensitization and changes in mu-receptor activity induced by repeated administration of amphetamine are illustrated in Figure 3. Exposure to argon during the withdrawal period led to an inhibition of locomotor sensitization to amphetamine. Indeed, when challenged with amphetamine, rats pretreated with amphetamine and argon had lower locomotor activity than control rats pretreated with amphetamine and air ($U = 41$, $P < 0.05$; Figure 3a). This indicated that argon had blocked the expression of locomotor sensitization to amphetamine. In contrast with its inhibitory effect on locomotor sensitization to amphetamine, argon had significant effect neither on the locomotor-activating action of acute amphetamine nor on basal locomotor activity (Figure 3a). Indeed, rats pretreated with saline solution and argon had locomotor activities that were not different from those displayed by rats pretreated with saline and air when challenged with amphetamine ($U = 20$, NS) or saline solution ($U = 17.5$, NS).

Exposure to argon during the withdrawal period blocked the increase in mu-receptor activity induced by repeated administration of amphetamine. Indeed, as assessed postmortem immediately after the amphetamine challenge, rats pretreated with amphetamine and argon had reduced mu-receptor activity compared with rats pretreated with amphetamine and air ($U = 0$, $P = 0.001$, Figure 3b). In contrast, as seen for locomotor activity, argon had effect on mu-receptor activity neither in rats pretreated with saline solution and challenged with amphetamine (acute amphetamine; $U = 30$, NS, Figure 3b), nor in control rats pretreated and challenged with saline solution ($U = 32$, NS, Figure 3b).

In vitro binding studies
Figure 4 illustrates the effects of argon on the binding of [³H] DAMGO. We found that the Bmax and Kd values, respectively, showed a decrease of 13% and an increase of 49% ($U = 5.5$, $P < 0.05$) in the presence of argon as compared with controls, which indicated that argon both reduced the number and affinity (1/Kd) of the mu-opioid receptor. This led to a reduction of 48% of the overall constitutive activity of the mu-receptor as assessed by the ratio of Bmax to Kd ($U = 3$, $P < 0.02$). Taken together, these data clearly demonstrated that argon has antagonistic properties at the mu-receptor.

Long-term sensitization studies
Repeated administration of amphetamine led to locomotor sensitization and context-dependent locomotor activity. Indeed, when challenged with amphetamine on day 58, rats pretreated with repeated amphetamine and then exposed to medicinal air during the withdrawal period had levels of locomotor activity that were significantly higher than those they displayed in response to their first amphetamine injection on day 1 ($Z = 2.192$, $P < 0.05$, Figure 5a). Also, in addition to locomotor sensitization, these rats further showed context-dependent locomotor activity. Indeed, as recorded on days 43–44 ($Z = -2.803$, $P < 0.005$) or 50–51 ($Z = -2.497$, $P < 0.02$; Figure 5a), these rats had higher score of basal locomotor activity when placed in the activity cages where they were usually pretreated with amphetamine than when placed in novel environmental activity cages.

Exposure to argon once or five times a week during the withdrawal period inhibited locomotor sensitization to amphetamine. Indeed, when challenged with amphetamine, rats pretreated with repeated amphetamine and then exposed to argon during the withdrawal period, once (Ar1) or five (Ar5) times a week, had levels of locomotor activity on day 58 that were not significantly different than those that they displayed in response to their first amphetamine injection on day 1 (Ar1: $Z = 0.784$, NS; Ar5: -0.078, NS; Figures 5b and c). However, although these rats did not express locomotor sensitization to amphetamine, they still showed context-dependent locomotor activity. Indeed, when recorded on days 43–44 (Ar1: $Z = -2.118$, $P < 0.05$; Ar5: -3.059, $P < 0.005$) or 50–51 (Ar1: $Z = -2.746$, $P < 0.01$; Ar5: -2.353, $P < 0.02$; Figures 5b and c), these rats had a higher score of basal locomotor activity, that is, when placed in the activity cages where they were usually pretreated with amphetamine than when placed in novel environmental activity cages.

DISCUSSION
Pharmacology of argon
The mechanisms by which the inert gases induce neuroprotection and further show therapeutic action are still little known. It seems, however, a common feature for the inert gases to act at multiple cellular targets. Xenon and nitrous oxide have facilitating effects at the TREK-1 potassium channel, as well as inhibiting effects on enzymes, such as tissue-plaminogen activator, the nicotinic
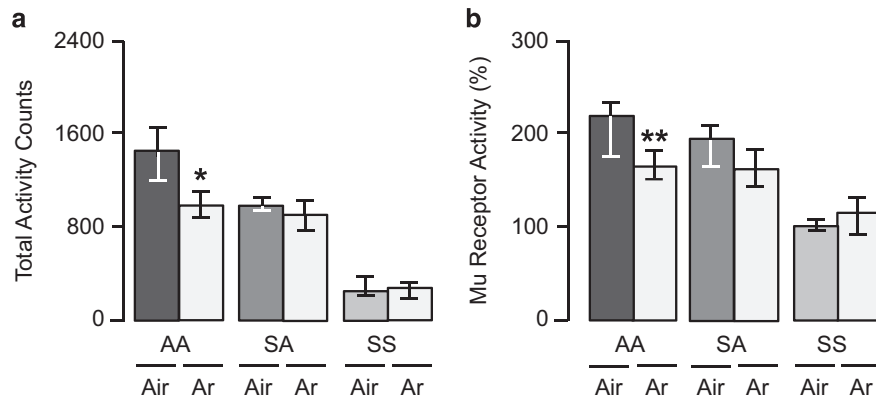
Figure 3. Effects of argon on amphetamine-induced changes in locomotor activity and mu-receptor activity in the nucleus accumbens. (**a**) When challenged with amphetamine, rats pretreated with amphetamine and argon had lower locomotor activity than rats pretreated with amphetamine and air (AA); in contrast, no significant difference in locomotor activity was found between rats pretreated with saline and argon and those pretreated with saline and air when challenged with amphetamine (SA) or saline (SS). This indicates that argon blocked locomotor sensitization to amphetamine, but had effect neither on locomotor activity induced by acute amphetamine nor on basal locomotor activity. Locomotor activity is expressed in arbitrary units. (**b**) As assessed postmortem immediately after being challenged with amphetamine, rats pretreated with argon and repeated administration of amphetamine (AA) had reduced mu-receptor constitutive activity in the nucleus accumbens (as estimated by the ratio of Bmax to Kd) compared with rats pretreated with air; in contrast, argon had effect on mu-receptor activity neither in rats pretreated with saline solution and challenged with amphetamine (SA) nor in control rats pretreated and challenged with saline solution (SS). This indicates that argon blocked the increase in mu-receptor activity induced by repeated administration of amphetamine, but had no effect on basal mu-receptor activity and acute amphetamine-induced changes in mu-receptor activity. Opioid mu-receptor activity in control rats pretreated and challenged with amphetamine was taken as a 100% value. Data are given as the median value $\pm$ 25th–75th percentiles; $n = 7$–8 per condition. $*P < 0.05$; $**P < 0.001$. AA, pretreatment+challenge with amphetamine; SA, pretreatment saline+challenge amphetamine; SS, pretreatment+challenge with saline.
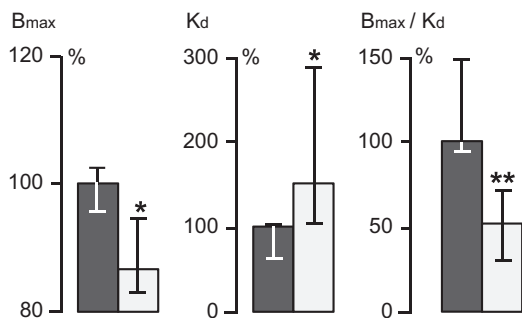

Figure 4. Effects of argon on the number of binding sites (Bmax) and dissociation constant (Kd) of the mu-opioid receptor in membrane preparations. (**a**) Argon caused a mild decrease in Bmax value of ~ 15% as compared with controls in the presence of nitrogen. (**b**) Argon increased Kd value by ~ 50%, thereby indicating a decrease of affinity (1/Kd). (**c**) Changes in Bmax and Kd values induced by argon led to a decrease in mu-opioid neurotransmission of $\approx$50% as estimated by the ratio of Bmax to Kd. Data are expressed as the median value $\pm$ 25th–75th percentiles; $n = 2$ per dose. $*P < 0.05$; $**P < 0.02$.

acetylcholine receptor, and mainly the *N*-methyl-D-aspartate glutamatergic receptor.[21–23,34] In contrast with these gases, argon has dual effects on tissue-plasminogen activator,[35] show no action on the *N*-methyl-D-aspartate receptor,[36] and has facilitating effects on the GABA-A and benzodiazepine receptors.[28] However, these latter effects were suggested from high pressure experiments, and so far no neuronal target had been identified for argon at atmospheric pressure. Therefore, by demonstrating *with in vivo* and *in vitro* binding assays that argon has antagonistic properties at the mu-opioid receptor both by reducing the number, and to a much greater extent, the affinity of this receptor, the present study provides a major advance in the pharmacology of argon.

In addition, we further found that argon blocked the amphetamine-induced increase in carrier-mediated dopamine

release and potentiated the amphetamine-induced decrease in KCl-evoked (depolarization-dependent) dopamine release. Amphetamine is a substrate for the dopamine transporter and the vesicular monoamine transporter-2,[37–41] which is considered an important pharmacological target for the treatment of amphetamine drug abuse.[42] Once bound, amphetamine increased carrier-mediated dopamine release by reversing the dopamine transporter,[41,43–45] and further reduced depolarization-dependent dopamine release attributable to synaptic vesicle exocytosis by redistributing dopamine from synaptic vesicles to the neuronal cytoplasm through inhibition of the vesicular monoamine transporter-2,[45–48] Interestingly, both amphetamine and the inert gases including argon are well known to penetrate cell membranes through lipophilic diffusion.[49–51] In addition, the inert gases also bind to proteins either within the active site(s) of the proteins or within hydrophobic pockets or cavities located close to the active site(s), thereby producing direct inhibition of protein function or conformational changes critical for protein function.[52–55] Given the inhibitory effects of argon on the amphetamine-induced increase in carrier-mediated dopamine release, it could be tempting to suggest that argon interacted directly, through a binding process, with the dopamine transporter. However, blocking the dopamine transporter with specific inhibitors has been shown not only to reduce the amphetamine-induced increase in carrier-mediated dopamine release but also to suppress the reduction in evoked dopamine release induced by amphetamine.[56] Though argon reduced the amphetamine-induced increase in carrier-mediated dopamine release, it further potentiated the decrease in KCl-evoked dopamine release induced by amphetamine, which indicates that argon is likely to be an inhibitor of the vesicular monoamine transporter-2.[57–59] However, changes in extracellular dopamine release and reuptake induced by amphetamines are known to be attenuated both in knockout mice lacking the mu-receptor and in rats treated with mu-receptor antagonists.[60–62] Therefore, it is possible that the antagonistic properties of argon at the mu-receptor shown in the present study could by themselves explain, at least partly, its inhibiting effect on the facilitating action of amphetamine on
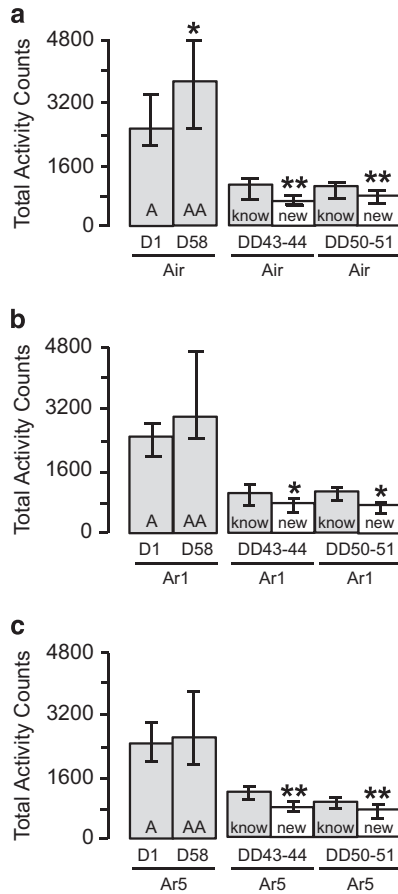
Figure 5. Effects of argon on long-term locomotor sensitization and context-dependent locomotor activity induced by repeated amphetamine administration. (**a**) Left: Pretreatment with repeated administration of amphetamine produced locomotor sensitization in response to an amphetamine challenge (AA) performed on day 58 compared with locomotor activity in response to acute amphetamine recorded on day 1. Right: Pretreatment with repeated administration in a given environment (see Materials and Methods section) produced context-dependent locomotor activity. Indeed, rats had a higher score of locomotor activity when placed on day 43–44 and 50–51 in the activity cages where they were given repeated administration of amphetamine (know) compared with their score of basal locomotor activity when placed in novel environmental activity cages (new). (**b**–**c**) Left: Argon at 75 vol% once a week (Ar1) (**b**) or five times a week (Ar5) (**c**) blocked locomotor sensitization to amphetamine. Right: In contrast, argon given once (Ar1) a week (**b**) or five times (Ar5) a week (**c**) had no effect on context-dependent locomotor activity induced by repeated administration of amphetamine. Data are expressed as the median value ± 25th–75th percentiles; $n = 10$–12 per condition. *$P < 0.05$; **$P < 0.02$.

carrier-mediated dopamine release. Support for this is the pharmacological profile of the natural alkaloid lobeline, which, in addition to its inhibiting properties at the vesicular monoamine transporter-2, also shows antagonistic properties at the mu-opioid receptor.[57–59,63]

Effects of argon on mu-receptor and locomotor sensitization to repeated amphetamine

The mu-opioid neurotransmission is an integral part of the motive circuit, and as such it is recognized to be fully involved in the mechanisms of action and the effects of drugs that belong to the amphetamine family.[6,32] We found that repeated administration of amphetamine led to a potentiation of the increase in mu-receptor

activity induced by acute amphetamine in the nucleus accumbens, a brain structure known to have a critical role in the behavioral effects of amphetamine and amphetamine-derived drugs.[1–3] These findings taken together with the inhibiting effect of amphetamine on KCl-evoked dopamine release, which indicates dopamine redistribution from synaptic vesicles to the neuronal cytoplasm, are in good agreement with in vitro data that have reported a concomitant dopamine accumulation and mu-receptor overexpression in neuroblastic dopaminergic cells treated with methamphetamine.[64] Also, they further agree with in vivo data that have shown an enhanced responsiveness and elevated constitutive activity of mu-opioid receptors in rats subjected to repeated administration of amphetamine.[65]

As expected from its antagonistic properties at the mu-receptor, we found that argon decreased the potentiating effect of repeated amphetamine on the increase in mu-receptor activity induced by acute amphetamine. However, argon had no effect on the increase in mu-receptor activity induced by acute amphetamine nor on basal mu-receptor activity. In line with these findings, we found that argon further blocked the expression of locomotor sensitization to amphetamine, but had effect neither on the increase in locomotor activity induced by acute amphetamine nor on basal locomotor activity. These effects of argon are in good agreement with previous findings that have demonstrated on one hand that knockout mice lacking the mu-receptor are insensitive to amphetamine-derived drug-induced behavioral sensitization,[32] and on the other hand that systemic administration of mu-receptor agonists enhances behavioral sensitization to amphetamine-derived drugs.[66] Also, they further agree with previous findings that have shown that lobeline, which pharmacological profile at the mu-opioid receptor and vesicular mono-amine transporter-2 is similar to that of argon,[57–59,63] attenuated the expression of locomotor sensitization to cocaine but failed to block the locomotor-activating properties of acute cocaine administration.[67]

Alternatively, argon has also been shown to produce narcosis at hyperbaric pressure through activation of the GABA-A receptor and benzodiazepine site.[28] Interestingly, in vivo studies have shown that repeated administration of methamphetamine inhibited the expression of the GABA-A receptor α2 subunit and GABA (A) receptor benzodiazepine [³H]flunitrazepam binding in the nucleus accumbens of mice.[68,69] Also, in vitro studies have further reported that repeated administration of methamphetamine decreased GABA-A receptor-evoked currents in xenopus ooxytes expressing the human α1β2γ2 GABA-A receptor.[70,71] In line with these data, other studies have shown that pharmacological compounds acting at the GABA-A receptor inhibited the discriminative stimulus effect of methamphetamine in mice and further reduced its use in humans.[72,73] Taken together these studies suggest that the agonistic properties of argon at the GABA-A receptor and benzodiazepine site could also contribute to its inhibiting action on locomotor sensitization to amphetamine, in addition of its antagonistic effects at the mu-opioid receptor and dopamine and monoamine transporters. In that way, because blocking the mu-receptor by specific antagonists inhibited the behavioral responses elicited by focal injection of GABA-A receptor agonists in the nucleus accumbens and other brain structures, in vivo studies have suggested that the GABA-A and mu-opioid receptors are closely linked.[74,75]

Effects of argon on long-term locomotor sensitization and context-dependent locomotor activity induced by repeated amphetamine administration

Addiction in humans occurs according to a long-term process of chronic consumption that includes periods of abstinence. Here we found, in a long-term model of sensitization and withdrawal relevant to these conditions, that argon suppressed the expression

of locomotor sensitization to amphetamine, but had no effect of the expression of the context-dependent locomotor activity induced by repeated administration of amphetamine. Given the antagonistic effects of argon at the mu-opioid receptor, the lack of effect of argon on the amphetamine-induced context-dependent locomotor activity could be viewed in contradiction with previous data that have demonstrated a critical role of the mu-receptor in spatial learning and memory, and further reported spatial learning and memory deficits in knockout mice lacking this receptor.[76,77] However, it should be noted that argon was given during the withdrawal period, 'far' from repeated pretreatment with amphetamine, a condition that could have avoided argon to impair spatial learning and memory. Also, an alternative explanation could be that amphetamine was given in activity cages that were kept in the dark and had sawdust on the floor, so that the context associated to amphetamine administration could have been more sensorial, that is, *stricto sensu* spatial.

Drug-induced locomotor sensitization and context-dependent locomotor activity are viewed, respectively, as models for the drug craving observed in human drug abusers and for the reward associated to the environmental stimuli related to drug intake. Therefore, our findings suggest that argon could suppress the craving for amphetamine and amphetamine-derived drugs in human drug abusers, but not the engram and memory trace associated to the environmental conditions of drug consumption. Although the latter suggests that argon as a potential treatment would not induce memory loss as an adverse side effect, it could be viewed as a condition that could favor relapsing in human drug abusers. Interestingly, however, it should be noted that previous data have shown that lobeline and its analogs, which pharmacological profile is similar to that of argon, decreased self-administration of methamphetamine and heroin in rats.[78–80] We suggest that self-administration studies could be done with argon using a long-term protocol similar to that used in the present study to determine whether relapsing would occur.

CONCLUSIONS

This study shows that argon blocked the expression of locomotor sensitization to amphetamine by inhibiting the mu-opioid receptor and vesicular monoamine transporter-2, whose critical role in drug addiction and dependence is well established. Given that no adverse effect has been reported in human subjects breathing 80 vol% argon at atmospheric pressure and in divers exposed to hyperbaric pressure equivalent to 300–600 vol% argon,[81–84] we believe that clinical trials with argon could be initiated safely in amphetamine-derived drug abusers. Interestingly, argon at 75 vol% further possesses prothrombolytic properties,[35] which could help in reducing the risk of vascular thrombosis in psychostimulant drug abusers,[85–88] in addition of its potential interest in the treatment of drug addiction.

CONFLICT OF INTEREST

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