The mammalian target of rapamycin pathway in the basolateral amygdala is critical for nicotine-induced behavioural sensitization

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Abstract
Repeated exposure to nicotine increases psychomotor activity. Long-lasting neural plasticity changes that contribute to the nicotine-induced development of locomotor sensitization have been identified. The mammalian target of rapamycin complex 1 (mTORC1) signalling pathway is involved in regulating the neuroplasticity of the central nervous system. In this study, we examined the role of mTORC1 in the amygdala in nicotine-induced locomotor sensitization. Rapamycin, an inhibitor of mTORC1, was infused into the basolateral amygdala (BLA) and central amygdala (CeA) or systemically administered to investigate the role of the mTORC1 in the development and expression of nicotine-induced locomotor sensitization. We found that locomotor activity progressively increased during the initiation of nicotine-induced locomotor sensitization and the expression of nicotine sensitization was induced by nicotine challenge injection (0.35 mg/kg s.c.) after five days of withdrawal. The initiation of nicotine-induced locomotor sensitization was accompanied by the increased phosphorylated level of mTORC1 downstream target proteins including p-p70s6k and p-4EBP in the BLA, but not CeA. Intra-BLA infusion or systemic administration of rapamycin blocked locomotor activity. Increased p-p70s6k and p-4EBP were also observed in the expression of nicotine sensitization, which was demonstrated to be inhibited by systemic rapamycin administration. Our findings indicated that mTORC1 activity in the BLA, but not the CeA, mediated the initiation and expression of nicotine-induced locomotor sensitization, and may become a potential target for the treatment of nicotine addiction.

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Introduction
Tobacco use kills more than five million people per year and is responsible for one in ten adult deaths. Among the five greatest risk factors for mortality, tobacco use is the single most preventable cause of death (WHO report on the global tobacco epidemic, 2013). Nicotine is the principal neuroactive component in tobacco, but the cellular mechanisms underlying its effects on behaviour remain unclear. Many abused drugs, including amphetamine, cocaine, opiates and nicotine, increase locomotor activity by activating brain dopamine (DA) neurotransmission in humans and laboratory animals (Vezina and Leyton, 2009). These effects are enhanced after repeated administration of drugs of abuse. Re-exposure to the drugs of abuse weeks to months after withdrawal produces greater dopaminergic and behavioural activation (Kalivas and Stewart, 1991; Vanderschuren and Kalivas, 2000; Vezina, 2004). This long-term enhancement in the ability of such drugs to activate DA neurotransmission and elicit appetitive behaviours is termed as sensitization, which may also contribute to the reinstatement of drug consumption in animals and drug craving in individuals after prolonged abstinence (Vezina, 2004; Leyton, 2007). Understanding the mechanisms of the initiation and expression of sensitization may be helpful in elucidating the neural events of nicotine addiction and how it may best be prevented.

Multiple studies on the mechanisms of nicotine-induced sensitization have focused on the mesolimbic dopamine system, which contributes to the rewarding effects of many addictive drugs, including nicotine. The amygdala is an important brain region involved in the mesocorticolimbic dopamine system for nicotine
addiction (Kryger and Wilce, 2010). High frequency basolateral amygdala stimulation has been reported to increase extracellular DA in the nucleus accumbens and induce NMDA and dopamine D1-dependent plasticity of basolateral amygdala inputs (Floresco et al., 1998, 2001). Furthermore, a recent study indicated that acute and sub-chronic nicotine induced c-fos activation in the BLA and nucleus accumbens shell of adolescent rats, whereas adults only showed nicotine-induced activation of BLA after sub-chronic treatment (Dao et al., 2011). This finding suggested that the BLA might be a critical brain region in nicotine sensitization.

The mammalian target of rapamycin (mTOR) kinase is an atypical serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins to form a complex named mTOR complex 1 (mTORC1) (Laplante and Sabatini, 2012). It plays a critical role in neuroplasticity by phosphorylating the downstream target proteins including p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding proteins (4EBPs) involved in the initiation and elongation phases of translation in neurons (Schratt et al., 2004; Tavazoie et al., 2005; Jaworski and Sheng, 2006; Parsons et al., 2006; Bekinschtein et al., 2007; Park et al., 2008). Phosphorylation of p70S6K and 4EBP is used as a marker of mTOR signalling. Previous studies have demonstrated that mTORC1 could be activated by NMDA and dopamine neurotransmitters (Lenz and Avruch, 2005; Gong et al., 2006) and involved in fear and drug-related memory (Bekinschtein et al., 2007; Blundell et al., 2008; Myskiw et al., 2008; Schicknick et al., 2008; Slipczuk et al., 2009; Barak et al., 2013; Lin et al., 2013). Additionally, mTORC1 activity in different brain regions played critical roles in drug-related behaviours, including cocaine or morphine-induced conditioned place preference or locomotor sensitization (Cui et al., 2010; Wu et al., 2011; Bailey et al., 2012), cue-induced reinstatement in non-humans and drug craving in heroin addicts (Shi et al., 2009; Wang et al., 2010; Barak et al., 2013). However, whether the activity of mTORC1 in the amygdala plays an important role in nicotine-induced sensitization is unclear. In the present study, we investigated the role of mTORC1 in the amygdala in nicotine sensitization and the effects of rapamycin, an inhibitor of mTORC1, which was infused into the basolateral amygdala (BLA), central amygdala (CeA) or systemically administered on the initiation and the expression of nicotine-induced locomotor sensitization.

Materials and Method

Subjects

Male Sprague-Dawley rats (n=187), weighing 220–250 g upon arrival, were housed in groups of four under controlled temperature (23±2 °C) and humidity (50±5%). The rats were maintained on a 12 h light/dark cycle (8:00–20:00) with ad libitum access to food and water and were accustomed to the animal house for five to seven days before the experiment. Rats weighed 300–320 g when experiments began. The experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Drugs

Nicotine (obtained from Sigma-Aldrich, 46343MSDS) was dissolved in 0.9% saline to a final concentration of 0.35 mg/ml and administered at a volume of 1 ml/kg, while the pH of the drug solution was adjusted to 7.3. Rapamycin (Sigma-Aldrich) was dissolved in 100% DMSO for intracranial injection and, for systemic administration, rapamycin was dissolved in vehicle that contained 4% anhydrous alcohol, 4% polyethylene glycol 2% Tween-80 (Blundell et al., 2008). The drugs were freshly prepared before use and the doses were based on previous reports (Li et al., 2008, 2011).

Surgery

Rats (weighing 300–320 g when surgery began) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Guide cannulas (23 gauge; Plastics One) were bilaterally implanted 1 mm above the basolateral and central amygdala. The respective coordinates (Paxinos et al., 1980) were as follows: anteroposterior (AP), −2.9 mm; mediolateral (ML), ±5.0 mm; dorsoventral (DV), −8.5 mm; and AP, −2.9 mm; ML, ±4.2 mm; DV, −0.8 mm. The surgical procedure was referenced from our previous reports (Lu et al., 2005; Li et al., 2008).

Intracranial injections

Rapamycin was infused bilaterally into the central or basolateral amygdala with Hamilton syringes connected to 30 gauge injectors (Plastics One). A total volume of 0.5 ul was infused bilaterally over 1 min, and the injector was kept in place for an additional 1 min to allow for diffusion. The doses of rapamycin were based on those of a previous study (Wang et al., 2010). At the end of the experiments, the rats were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and transcardially perfused. Cannula placements were assessed using Nissl staining with a thickness of 40 mm under light microscopy. Subjects with misplaced cannulas (approximately 2%) were excluded from statistical analysis.

Tissue sample preparation

Rats (also including naïve rats that had no drug history or behavioural history and were kept in the home
cage during the experiments) were rapidly decapitated without anesthesia 1 h after being subcutaneously injected with nicotine. After decapitation, bilateral tissue in the central or basolateral amygdala were micro-punched and prepared for the determination of protein concentrations via Western blot assay according to our previous studies (Li et al., 2008).

### Western blot assays

The samples were treated according to our previous studies (Lu et al., 2005, 2008, 2011). Samples were subjected to SDS-PAGE (10% acrylamide/0.27% N, N-methylenebisacrylamide resolving gel) for ~30 min at 80 V in stacking gel and ~1 h at 120 V in resolving gel. Proteins were transferred electrophoretically to immobilon-P transfer membranes (Millipore) at 0.25 A for 3 h. Membranes were washed with TBST (Tris-buffered saline plus 0.05% Tween 20, pH 7.4) and then dipped in blocking buffer (5% skimmed dry milk in TBST) overnight at 4 °C. The next day, the membranes were incubated for 1 h at room temperature on an orbital shaker with anti-p-p70s6k antibody (1:500; Santa Cruz) and anti-p-4EBP antibody (1:500; Santa Cruz), anti-t-p70s6k antibody (1:500; Santa Cruz) and anti-t-4EBP antibody (1:500; Santa Cruz), or anti-β-actin(1:500; Santa Cruz) in TBST plus 5% BSA and 0.05% sodium azide. After four six-minute washes in TBST buffer, the blots were incubated for 45 min at room temperature on a shaker with horseradish peroxidase (HRP)-conjugated secondary antibody (sheep anti-rat IgG; Santa Cruz) diluted 1:5000 in blocking buffer. The blots were then washed four times for six minutes in TBST and then incubated with a layer of a mix of Super Signal Enhanced chemiluminescence (ECL) substrate (Detection Reagents 1 and 2 at a 1:1 ratio; Pierce) for 1 min at room temperature. Excess mix was dripped off before blots were wrapped with a clean piece of Saran Wrap (no bubbles between blot and wrap) and then exposed against x-ray film (Eastman Kodak) for 5–60 s. Band intensities for t-p70s6k, t-4EBP and p-p70s6k, p-4EBP were quantified by two observers (blind to experimental groups) using Quantity One software (version 4.4.0) from Bio-Rad. Band intensities from each test sample were compared with the band intensities from the standard curves. The amount of the protein of interest in the samples was interpolated from standard curves. Only the data for t-p70s6k, t-4EBP and p-p70s6k, p-4EBP were presented because β-actin was not significantly different between any of the groups in the present study.

### Behavioural sensitization

The experimental procedure for behavioural sensitization was performed according to previous studies (Xu et al., 2009), consisting of four phases: adaptation; induction; withdrawal; and expression.

### Adaptation phase

The rats were placed into the chambers for 60 min to monitor locomotor activity after daily injections of 0.9% saline (1 ml/kg s.c.) for three days. The level of locomotor activity on the last adaptation day (day 0) was defined as baseline.

### Initiation phase

Locomotor activity was monitored for 60 min after a daily injection of 0.9% saline (1 ml/kg s.c.) or nicotine (0.35 mg/kg s.c.) for five days. The experiments began at 08:00 h and the injections were performed at the same time each day.

### Withdrawal phase

The rats were kept in home cage for seven days with food and water freely available.

### Expression phase

After seven days of withdrawal in the home cage, locomotor activity was again monitored for 1 h following a single nicotine challenge (0.35 mg/kg s.c.) to observe the expression of nicotine-induced sensitization. The timing of the tests on the test day was same as the timing on the training days in each group of rats.

### Locomotor activity test

Locomotor activity was measured with an automated video tracking system (DigBehv-LM4, Shanghai Jiliang Software Technology, China) that contained eight identical clear Plexiglas chambers (40×40 ×65 cm). The video files were analysed using DigBehv analysis software. Locomotor activity was expressed as the total distance (mm) travelled during a predetermined period of time (min).

### Study design

**Experiment 1 – Activation of mTORC1 in the basolateral or central amygdala during the initiation of nicotine sensitization**

To examine the activation of mTORC1 in the basolateral or central amygdala during the initiation of nicotine sensitization, the phosphorylation of its downstream targets including p70S6K and 4EBPs were tested. Rats were divided into two groups (n=14–16 per group) and given nicotine (0.35 mg/kg s.c.) or 0.9% saline (1 ml/kg s.c.) injections every day, followed by 1 h of locomotor monitoring for 5 consecutive days (Fig. 1a). On the last day, the rats were decapitated immediately for p70s6k and 4EBP phosphorylation determination in the basolateral or central amygdala after 1 h locomotor monitoring.
Experiment 2 – Effects of rapamycin intracranial infusion into basolateral or central amygdala on initiation of nicotine sensitization

The results of experiment one demonstrated that mTORC1 activation in the BLA but not the CeA was induced by nicotine, but they did not show whether the BLA or CeA is required for the initiation of nicotine sensitization. To examine its critical role in the initiation of nicotine sensitization in the BLA or CeA, we used eight groups of rats (n=14–16 per group) who received bilateral micro-injections of rapamycin (50 μg/side, 0.5 ul) or vehicle (0.5 ul/side) into the BLA or CeA 30 min before nicotine or saline injections every day, and then distance travelled was tested for 5 consecutive days (Fig. 2a and 3a). On the last day, the rats were decapitated immediately after locomotor monitoring to detect whether mTORC1 activity was inhibited by rapamycin in BLA or CeA via Western blot assay.

Experiment 3 – Effects of systemic rapamycin administration on the initiation of nicotine sensitization

Rapamycin is a FDA-approved drug used for the prevention of the host rejection of organ transplants. To determine the potential possibility that the inhibitory effect of rapamycin for nicotine sensitization could be utilized in therapy, we used another eight groups of rats (n=14–16 per group) who received systemic administration of rapamycin (0, 1, 3, 10 mg/kg) 45 min before nicotine (0.35 mg/kg s.c.) or 0.9% saline (1 ml/kg s.c.) injections every day, followed by 1 h of locomotor monitoring for five consecutive days (Fig. 4a). On the last day, the rats in each group after drug administration were divided into two groups: one group to receive monitoring of locomotor activity, and the other for examination of p70s6k and 4EBP phosphorylation in the basolateral or central amygdala via Western blot assay.

Experiment 4 – Activation of mTORC1 in the basolateral or central amygdala during the expression of nicotine sensitization

To determine the activation of mTORC1 of in the basolateral or central amygdala during the expression of nicotine sensitization, we used four groups of rats (n=14–16 per group) who received a challenge with the systemic administration of nicotine (0.35 mg/kg s.c.) or 0.9% saline (1 ml/kg s.c.) injection 5 days after nicotine withdrawal. Then half of the rats underwent a 1-h locomotor activity test, while the other rats were decapitated and their brains were extracted for the subsequent determination of mTORC1 activity in the basolateral or central amygdala by Western blot (Fig. 5a).
Experiment 5 – Effects of systemic rapamycin administration on the expression of nicotine sensitization

To determine whether rapamycin has an effect on the expression of nicotine sensitization, four groups of rats (n=14–16 per group) received daily injections of nicotine (0.35 mg/kg s.c.) or 0.9% saline (1 ml/kg i.p.), followed by 1 h of locomotor activity monitoring for 5 consecutive days. Based on the results of the above experiment, the rats received an effective dose of rapamycin (10 mg/kg, i.p.) or vehicle 5 days after nicotine withdrawal. Then, half of the rats underwent a locomotor activity test, while the others were used to determine the mTORC1 activity in the basolateral or central amygdala by Western blot (Fig. 6a).

Statistical analysis

The data analysis was referenced from previous reports (Koek, 2014; Sun et al., 2013). Data were expressed as the mean±S.E.M and were analysed by analysis of variance (ANOVA). For the behavioural test data, repeated-measures ANOVA analyses were conducted with treatment (nicotine injection and rapamycin...
administration) as the between-subjects factor and test day (day 1, 2, 3, 4 and 5 or 10) as the within-subjects factor for each of the experiments (see Results section). For Western blot of p-p70s6k and 4EBP levels analysis, data were transformed as a percentage of control from ‘naïve’ animals, ANOVA was conducted, with the cranial infusion (0 and 50 μg/side)/systemic administration (0, 1, 3 and 10 mg/kg) of rapamycin and nicotine (0 and 0.35 mg/kg) as the between-subjects factors. All the post-hoc comparisons were made using Tukey’s test. Values of p<0.05 were considered statistically significant.

For clarity, significance in the post-hoc analysis was indicated by symbols in the figures.

**Results**

**Experiment 1 – Activation of mTORC1 in the basolateral or central amygdala during the initiation of nicotine sensitization**

Repeated-measures ANOVA was used in nicotine-induced locomotor activity testing with the nicotine
dose (0 and 0.35 mg/kg) as the between-subjects factor and the test day (day 1, 2, 3, 4 and 5) as the within-subjects factors. As shown in Fig. 1, repeated-measures ANOVA revealed that there were significant effects of nicotine dose ($F_{1,14} = 12.34, p < 0.01$), and test day ($F_{4,56} = 5.21, p < 0.01$) and an interaction between nicotine dose x test day ($F_{4,56} = 13.09, p < 0.05$). Post-hoc analysis showed a significant difference in distance between saline- and nicotine-injected rats on each day from day one to day five (Fig. 1b). For nicotine-induced p70s6k and 4EBP phosphorylation, one-way ANOVA revealed a significant effect of nicotine dose in BLA ($F_{1,15} = 13.59, p < 0.01$; $F_{1,15} = 43.17, p < 0.01$, respectively), but not in CeA ($F_{1,15} = 0.02, p > 0.05$; $F_{1,15} = 0.00, p > 0.05$, respectively) (Fig. 1c, d). Moreover, no significant effect of nicotine on the total p70s6k level and 4EBP was found in either the basolateral
(F\(_{1,15}=0.03, \ p>0.05\); (F\(_{1,15}=0.01, \ p>0.05\), respectively) or central (F\(_{1,15}=0.01, \ p>0.05\); F\(_{1,15}=0.08, \ p>0.05\), respectively) amygdala between groups. These results demonstrated that nicotine progressively increased locomotor activity and mTORC1 activity in the basolateral but not central amygdala of rats.

**Fig. 5.** Effects of nicotine challenge on the phosphorylation of P70S6K and 4EBP in the basolateral or central amygdala during the expression of nicotine sensitization. (a) Behavioural procedure. (b) Expression of locomotor sensitization was induced by nicotine challenge. Locomotor activity was increased by nicotine challenge and monitored for 1 h after injection of saline or nicotine (0.35 mg/kg s.c.) 5 days after withdrawal. Locomotor activity data on day 0 served as baseline. The data are expressed as the mean±S.E.M. (n=8). (c–d). Phosphorylated and total P70S6K and 4EBP in BLA (c) and CeA (d) in the rats that received nicotine or saline challenge (0.35 mg/kg, s.c.). Data are expressed as a percentage (mean±S.E.M.) of those of naïve rats. * p<0.05 relative to saline challenge group.
Experiment 2 – Effects of rapamycin intracranial infusion into basolateral or central amygdala on initiation of nicotine sensitization

ANOVA was used to analyse locomotor activity and included the between-subjects factors of rapamycin (0 and 50 ug/side) and nicotine (0 and 0.35 mg/kg) and within-subjects factor of test day (day 1, 2, 3, 4 and 5). For administration of rapamycin into BLA, the ANOVA revealed significant effects of nicotine ($F_{1,28}=23.65, p<0.05$), rapamycin ($F_{1,28}=7.23, p<0.05$), and test day ($F_{1,28}=8.79, p<0.05$), a significant interaction between nicotine × test day ($F_{4,28}=4.68, p<0.05$), and a significant interaction among nicotine × rapamycin × test day.
effects of nicotine (0.35 mg/kg) and rapamycin (0, 10 mg/kg). The analysis revealed significant effects of nicotine (F_{1,28}=17.28, p<0.05) and test day (F_{1,28}=16.69, p<0.05) and a significant interaction between nicotine x test day (F_{1,28}=11.27, p<0.05), but no main effect of rapamycin (p>0.05) and no nicotine x rapamycin x test day interaction (p>0.05) (Fig. 3b). Phosphorylation of p70s6K in basolateral or central amygdala determined by Western blot assay were measured with mixed ANOVA. Rapamycin (0 and 50 μg/side) and nicotine (0 and 0.35 mg/kg) served as the between-subjects factors. The analysis of the data from the Western blot assays revealed that BLA and CeA infusions of rapamycin decreased p-p70s6K (F_{1,28}=8.62, p<0.01, and F_{1,28}=12.34, p<0.01, respectively) and p-4EBP (F_{1,28}=19.31, p<0.01, and F_{1,28}=56.32, p<0.01, respectively) (Fig. 2c and 3c). The total p70s6K (F_{1,28}=1.65, p>0.05, and F_{1,28}=1.21, p>0.05, respectively) and 4EBP (F_{1,28}=0.31, p>0.05, and F_{1,28}=0.24, p>0.05, respectively) protein levels in the BLA and CeA were not significantly affected in any of the rats (Fig. 2c and 3c). However, rapamycin infusion into the BLA but not the CeA inhibited the initiation of nicotine sensitization.

**Experiment 3 – Effects of systemic rapamycin administration on the initiation of nicotine sensitization**

The initiation of nicotine sensitization was inhibited by the systemic administration of rapamycin (10 mg/kg, i.p.) 45 min before each daily nicotine injection (Fig. 4b). Locomotor activity was assessed using repeated-measures ANOVA including the between-subjects factors of nicotine (0 and 0.35 mg/kg) and rapamycin (0, 1, 3 and 10 mg/kg), and the within-subjects factor of test day (days 1, 2, 3, 4, and 5). The analysis revealed significant effects of nicotine (F_{1,56}=165.52, p<0.05), rapamycin (F_{3,56}=17.24, p<0.05), and test day (F_{4,56}=14.28, p<0.05), a significant interaction between nicotine x test day (F_{1,224}=21.82, p<0.05), a significant interaction between rapamycin x test day (F_{1,224}=12.65, p<0.05), and a significant interaction among nicotine x rapamycin x test day (F_{12,224}=13.20, p<0.05). Nicotine induced locomotor sensitization accompanied increased p-p70s6K and 4EBP in the BLA, but not in the CeA (Fig. 4c). Rapamycin blocked the increase in p-p70s6K and 4EBP in the BLA (Fig. 4c). The phosphorylation of p70s6K and 4EBP were analysed separately for BLA or CeA using mixed ANOVA including the between-subjects factors of nicotine (0 and 0.35 mg/kg) and rapamycin (0, 10 mg/kg). The analysis of p-p70s6K and p-4EBP levels in BLA revealed significant effects of nicotine (F_{1,28}=32.51, p<0.05; F_{1,28}=54.32, p<0.05, respectively) and rapamycin (F_{1,28}=27.12, p<0.05; F_{1,28}=101.5, p<0.05, respectively) and a significant interaction between nicotine x rapamycin (F_{1,28}=36.13, p<0.05; F_{1,28}=114.27, p<0.05, respectively). The ANOVA for p-p70s6K and p-4EBP levels in the CeA revealed no significant interaction between the two factors (F_{1,28}=0.13, p>0.05; F_{1,28}=0.27, p>0.05, respectively). Total p70s6k (F_{1,28}=0.29, p>0.05; F_{1,28}=0.58, p>0.05, respectively) and 4EBP (F_{1,28}=0.01, p>0.05; F_{1,28}=0.007, p>0.05, respectively) protein levels in the BLA and CeA did not significantly change in any of the groups (Fig. 4c).

**Experiment 4 – Activation of mTORC1 in the basolateral or central amygdala during the expression of nicotine sensitization**

ANOVA revealed significant effects of nicotine initiation dose (F_{1,14}=127.11, p<0.05), nicotine challenge dose (F_{1,14}=22.46, p<0.05), and a significant interaction between nicotine initiation dose x nicotine challenge dose (F_{4,14}=19.84, p<0.05). Nicotine challenge-induced locomotor sensitization accompanied with the increased p-p70s6K and p-4EBP in the BLA, but not the CeA (Fig. 5c and 5d). Analyses were performed separately for p-p70s6K and p-4EBP levels in the BLA and CeA using one-way ANOVA. The ANOVA for p-p70s6K and p-4EBP levels in the BLA revealed significant effects of nicotine challenge (F_{1,15}=15.01, p<0.05; F_{1,15}=102.31, p<0.05, respectively) (Fig. 5c). The ANOVA for p-p70s6K levels and p-4EBP in the CeA revealed no significant effects of nicotine challenge (F_{1,15}=0.002, p>0.05; F_{1,15}=0.008, p>0.05, respectively) (Fig. 5d). The total p70s6k (F_{1,15}=0.001, p>0.05; F_{1,15}=0.072, p>0.05, respectively) and 4EBP (F_{1,15}=0.01, p>0.05; F_{1,15}=0.007, p>0.05, respectively) protein levels in the BLA or CeA were unaffected (Fig. 5c and d).

**Experiment 5 – Effects of systemic rapamycin administration on the expression of nicotine sensitization**

The ANOVA revealed significant effects of nicotine (F_{1,28}=117.44, p<0.05) and test day (F_{1,28}=34.41, p<0.05) and a significant interaction between nicotine x test day (F_{1,28}=43.11, p<0.05). Figure 6b showed that following systemic nicotine (0.35 mg/kg, s.c.) challenge on day 10 after withdrawal, the locomotor activity was inhibited by rapamycin (10 mg/kg, i.p.). Nicotine (0 and 0.35 mg/kg, s.c.) and rapamycin (0 and 10 mg/kg) served as the between-subjects factors. The ANOVA revealed significant effects of nicotine (F_{1,28}=18.32, p<0.05) and rapamycin (F_{1,28}=32.89, p<0.05) and a significant nicotine x rapamycin (F_{1,28}=9.91, p<0.05) (Fig. 6b). The expression of locomotor sensitization after nicotine challenge accompanied increased p-p70s6K and p-4EBP levels in the BLA, but not the CeA. The systemic administration of rapamycin reduced the increased p-p70s6K and p-4EBP levels in the BLA. The analysis was performed separately for p-p70s6K and p-4EBP levels in the BLA using mixed ANOVA. The ANOVA for p-p70s6K and p-4EBP levels in the BLA revealed significant effects of nicotine (F_{1,28}=21.09, p<0.05; F_{1,28}=131.14, p<0.05, respectively) and rapamycin (F_{1,28}=19.31, p<0.05; F_{1,28}=69.24, p<0.05,
respectively) and a significant nicotine×rapamycin interaction ($F_{1,28}=15.24, p<0.05$; $F_{1,28}=131.14, p<0.05$, respectively) (Fig. 6c). The experimental manipulations had no effect on the total p70s6k and 4EBP protein levels in the BLA ($F_{1,28}=0.32, p>0.05$; $F_{1,28}=0.19, p>0.05$, respectively) (Fig. 6c).

Discussion

The present study indicated that mTORC1 activity in the basolateral amygdala played a critical role in the initiation and expression of nicotine-induced sensitization. The main findings of the present study were: (i) locomotor activity was progressively increased by five days of consecutive nicotine administration and a nicotine challenge after five days of withdrawal (these behavioural changes were accompanied by increased p-p70s6k and p-4EBP in the BLA, but not the CeA); and (ii) the inhibition of mTORC1 activity by the systemic administration of rapamycin or intra-BLA injection inhibited the initiation and expression of nicotine-induced behavioural sensitisation. These findings suggested a novel role of the mTORC1 activity in the BLA for nicotine sensitization and the potent therapeutic value of rapamycin in nicotine addiction.

Role of the basolateral amygdala in nicotine-induced sensitization

Studies in an animal model and in smokers using neuroimaging indicated that the amygdala is activated by nicotine administration or withdrawal (Mihov and Hurlemann, 2012). Drug behavioural sensitization is most likely mediated by the mesocortical and mesoamygdaloid dopamine projections combined with glutamate transmission (Pierce and Kalivas, 1997). The BLA receives strong glutamatergic innervation from the thalamus, hippocampus and medial prefrontal cortex (Ottersen, 1982; Albanese and Minciachic, 1983; van Vulpen and Verwer, 1989), and it also receives dopaminergic innervation from the VTA (Albanese and Minciachic, 1983), which has an integral role in processing affective states (Phelps and LeDoux, 2005). Moreover, abundant evidence indicates that nicotinic acetylcholine receptors (nAChR) are densely distributed in the mesocorticolimbic dopamine system (De Biasi and Dani, 2011) and functional nAChRs expressed on pyramidal cells (somatodendritic $\alpha 7$), GABAergic interneurons ($\alpha 7$, $\alpha 4\beta 2$, and $\alpha 3\beta 4$), and glutamatergic afferents ($\alpha 7$) that modulate synaptic transmission in the amygdala (Hill et al., 1993; Klein and Yakel, 2006; Perry et al., 2007). Different pathways within the BLA are responsible for various functions in the initiation and expression of behavioural sensitisation to repeated drug administration mediated by nicotinic acetylcholine receptors (Stamatakis et al., 2014). Therefore, the BLA may play a critical role in drug sensitization induced by nicotine (Stamatakis et al., 2014). In our present study, we demonstrated that mTORC1 activity in the BLA, but not in the CeA, increased in the initiation and expression of nicotine-induced behavioural sensitization. The local delivery of rapamycin into the BLA inhibited the nicotine-induced behavioural sensitization. These results further extended the role of BLA in drug sensitization.

Mammalian target of rapamycin involved in nicotine-induced sensitization

It is well known that long-lasting drug-induced neuroplasticity changes in the brain may underlie persistent behaviours such as sensitization, tolerance and relapse (Robinson and Berridge, 2003; Kalivas and Volkow, 2005; Hyman et al., 2006; Russo et al., 2010). A previous study found that up-regulated nAChRs in the BLA induced by long-term nicotine administration play a critical role in the development of this behavioural response (Parker et al., 2004). Also, several in vitro studies have indicated that nicotine stimulates lung cancer cell growth through the nAChR mediated PI3K/mTOR signalling pathway (Tsurutani et al., 2005; Zheng et al., 2007; Sun et al., 2009). In our present study, we found that the activity of mTORC1 was activated by nicotine both in the initiation and expression of sensitization in BLA, which indicated that mTORC1 might be activated by nicotine via nAChRs. Neuroadaptation regulated by the mTORC1 signalling pathway in the basolateral amygdala is required for different processes of fear memory, including acquisition, consolidation and reconsolidation (Takeuchi et al., 1982; Bekinschtein et al., 2007; Blundell et al., 2008; Slipczuk et al., 2009; Barak et al., 2013), as well as stress- and reward-related behaviours (Simpson et al., 2000; Stuber et al., 2011; Myers-Schulz and Koenigs, 2012). Additionally, in addiction, mTORC1 activity is required for cue-induced relapse both in human and animal studies (Shi et al., 2009; Wang et al., 2010; Barak et al., 2013). This findings suggested that the mTORC1 signalling pathway in BLA was a critical molecular for the neuroadaptation involved in different neuronal process. The result in our present study that the neuroplasticity mediated by mTORC1 activity in the BLA but not CeA was required for the initiation and expression of nicotine-induced behavioural sensitisation extended its role in drug abuse.

Rapamycin blocked the initiation and expression of nicotine-induced sensitization and its potent therapeutic potential

Rapamycin is a highly specific inhibitor of mTORC1 (Davies et al., 2000; Bodine, 2006). In previous studies, rapamycin has been used to investigate the role of mTORC1 in memory formation and reconsolidation (Blundell et al., 2008). In the present study, our results showed that the microinjection of rapamycin in the basolateral, but not central amygdala, caused a significant decrease in locomotor activity in the development and
expression of nicotine-induced sensitization by inhibiting the levels of p70s6k phosphorylation. This inhibitory effect was also observed in the systemic administration of rapamycin in a dose-dependent way for the initiation and expression of nicotine-induced sensitization. Rapamycin is a FDA-approved drug that is used for the prevention of the host rejection of organ transplants (Hartford and Ratain, 2007). Moreover, in our study, the microinjection of rapamycin decreased the level of phosphorylation in the BLA and CeA. The systemic administration of rapamycin only decreased the increased phosphorylation of p70s6k and 4EBP1 in BLA in a dose-dependent way, but didn’t change the normal level of them. And the dosage of 10 mg/kg of rapamycin used in the present study did not change the locomotor activity in rats, which have been observed in our previous study (Lin et al., 2013). Therefore, these findings put forward the possibility that by inhibiting the mTORC1 signalling cascade in the brain by rapamycin to be an innovative and valuable treatment strategy for nicotine dependence.

In summary, mTORC1 activity in the BLA plays a critical role in the development of nicotine-induced sensitization. Rapamycin, an inhibitor of mTORC1, may dampen the initiation and expression of nicotine-induced sensitization. Our findings may have important implications for understanding the neurobiological mechanism for nicotine-induced sensitization and rapamycin may be a potential drug for the treatment of nicotine addiction.

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Statement of Interest

None.

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