**ORIGINAL ARTICLE**

**Sustained AAV-mediated overexpression of CRF in the central amygdala diminishes the depressive-like state associated with nicotine withdrawal**

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Smoking cessation leads to a dysphoric state and this increases the risk for relapse. Animal studies indicate that the dysphoric state associated with nicotine withdrawal is at least partly mediated by an increase in corticotropin-releasing factor (CRF) release in the central nucleus of the amygdala (CeA). In the present study, we investigated whether a sustained overexpression of CRF in the CeA affects the dysphoric-like state associated with nicotine withdrawal. To study brain reward function, rats were prepared with intracranial self-stimulation (ICSS) electrodes in the medial forebrain bundle. An adeno-associated virus (AAV, pseudotype 2/5) was used to overexpress CRF or green fluorescent protein (GFP, control) in the CeA and minipumps were used to induce nicotine dependence. The AAV2/5-CRF vector induced a 40% increase in CRF protein and mRNA levels in the CeA. Administration of the nicotinic receptor antagonist mecamylamine (precipitated withdrawal) or nicotine pump removal (spontaneous withdrawal) led to elevations in ICSS thresholds. Elevations in ICSS thresholds are indicative of a dysphoric-like state. The overexpression of CRF did not affect baseline ICSS thresholds but diminished the elevations in ICSS thresholds associated with precipitated and spontaneous nicotine withdrawal. The real-time reverse transcriptase (RT)–PCR analysis showed that the overexpression of CRF led to a decrease in CRF1 mRNA levels and an increase in CRF2 mRNA levels in the CeA. In conclusion, the overexpression of CRF in the CeA diminishes the dysphoric-like state associated with nicotine withdrawal and this might be driven by neuroadaptive changes in CRF1 and CRF2 receptor gene expression.

Translational Psychiatry (2014) 4, e385; doi:10.1038/tp.2014.25; published online 22 April 2014

**INTRODUCTION**

Tobacco addiction is characterized by a loss of control over smoking, withdrawal symptoms, and relapse.1–3 Smoking cessation leads to dysphoria and increased anxiety and these negative affective symptoms provide a powerful incentive for the continuation of smoking.4–7 Animal studies suggest that the activation of brain stress systems contributes to the negative affective state associated with nicotine withdrawal.6

Corticotropin-releasing factor (CRF) was first isolated from the hypothalamus and shown to regulate hypothalamic–pituitary–adrenal axis activity.8 Follow-up studies showed that CRF is expressed in a variety of extrahypothalamic brain sites and mediates behavioral effects independent of its effects on the hypothalamic–pituitary–adrenal axis.9,10 It has now been established that CRF systems are dysregulated in a variety of psychiatric disorders, including depression, anxiety disorders, and drug addictions.11,12 Corticotropin-releasing factor mediates its effects on emotional states via the activation of CRF1 and CRF2 receptors. Corticotropin-releasing factor is the main endogenous ligand for the CRF1 receptor and mediates anxiogenic and depressive-like effects via the activation of this receptor.13 Corticotropin-releasing factor also binds to CRF2 receptors but with a much lower affinity.14 The urocortins (urocortin 1–3) bind to the CRF2 receptor with a high affinity and are considered the endogenous ligands for this receptor.15–16 Studies with selective drugs and knockout mice indicate that CRF2 receptor activation opposes the effects of stress and has anxiolytic and antidepressant-like effects.17–19

There is accumulating evidence for a role of CRF in the rewarding effects of nicotine and nicotine withdrawal. Stressors facilitate the initiation of smoking and animal studies indicate that stressors potentiate the rewarding effects of nicotine via a CRF1 receptor dependent mechanism.6,20 Blockade of CRF1 receptors diminishes nicotine withdrawal-induced anxiety-like behavior.21 In addition, nicotine withdrawal leads to an increase in CRF in the central nucleus of the amygdala (CeA) and blockade of CRF1 receptors in this brain site prevents high levels of nicotine intake.21,22 Our laboratory explored the role of CRF in the dysphoric-like state associated with nicotine withdrawal. We showed that blockade of CRF1 receptors in the whole brain or in the CeA diminishes the dysphoric-like state associated with precipitated nicotine withdrawal.23,24 Taken together, the nicotine withdrawal-induced increase in CRF release in the CeA leads to a negative affective state which contributes to increased nicotine intake. It is interesting to note that recent studies have shown that a brief period of lentiviral-mediated CRF overexpression increases stress-induced anxiety-like behavior whereas prolonged overexpression of CRF dramatically decreases stress-induced anxiety-like behavior in mice.25,26 This suggests that chronic overexpression of CRF may lead to the adaptations that diminish the effects of CRF. Therefore, this would suggest that prolonged

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Received 11 October 2013; revised 10 January 2014; accepted 8 March 2014
overexpression of CRF could potentially diminish the dysphoric-like state associated with nicotine withdrawal.

Many questions regarding the role of CRF in nicotine addiction remain unanswered. In particular, it is unknown if a prolonged increase in CRF levels in the CeA affects brain reward function in drug naïve rats and nicotine-withdrawing rats. Therefore, these studies investigated the effects of chronic adeno-associated virus (AAV2/5)-mediated overexpression of CRF in the CeA on baseline intracranial self-stimulation (ICSS) thresholds (28 days) and ICSS thresholds during precipitated and spontaneous nicotine withdrawal. In addition, we assessed CRF₁ and CRF₂ gene expression levels in the CeA to gain insight into the mechanisms by which the overexpression of CRF might affect nicotine withdrawal.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats (200–225 g, Charles River, Raleigh, NC, USA) were housed (two per cage) in a climate-controlled vivarium on a reversed 12 h light-dark cycle. Food and water were available ad libitum. The experimental protocols were approved by the UF Institutional Animal Care and Use Committee.

Production and validation of AAV2/5 vectors

The AAV2/5-CRF vector was obtained from Vector BioLabs (Philadelphia, PA, USA) and AAV2/5-GFP from the UF Powell Gene Therapy Center (Gainesville, FL, USA). Rat CRF cDNA (GenBank accession number NM_031019) and humanized green fluorescent protein (GFP) cDNA were packaged into the AAV2/5 vector. The transduction efficiency of the vectors was evaluated by assessing transgene expression in primary neuronal cultures and the brain (see also Supplementary Methods).

ICSS procedure and microinjections

The rats were prepared with an electrode in the medial forebrain bundle and bilateral cannulae above the CeA using coordinates from the Paxinos and Watson rat brain atlas. The animals were trained on the ICSS procedure until the thresholds were stable (≤ 10% variation over a 5-day period). Each test session provided an ICSS threshold and response latency. Elevations in ICSS thresholds reflect a negative mood state. When the ICSS thresholds were stable, AAV2/5-GFP or AAV2/5-CRF was infused bilaterally into the CeA. Surgeries, ICSS testing, and CeA infusions were done as described before (see also Supplementary Methods).

Immunohistochemistry, real-time reverse transcriptase (RT)–PCR, and western blotting

For the immunohistochemistry, the animals were perfused and 40 μm coronal brain sections were cut on a cryostat. The sections were incubated with antibodies, mounted on slides, and examined with a confocal microscope. To assess CRF and CRF receptor mRNA levels, RNA was isolated from harvested primary neuronal cultures or micropunched brain tissues. Then CRF, CRF₁, and CRF₂ receptor mRNA levels were determined using real-time RT–PCR. Corticotropin-releasing factor protein levels were determined by western blotting. The CeA was micropunched and homogenized and proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated with CRF antibodies and visualized on film (see also Supplementary Methods).

Statistics

The data were analyzed with ANOVAs (analyses of variance) or Student’s t-tests. Significant findings in the ANOVAs were followed by Newman–Keuls post hoc tests (see also Supplementary Methods).

Experimental design

Experiment 1: Time course and tropism of AAV2/5-mediated GFP expression in CeA. The viral vector (AAV2/5-GFP, n = 18) or sterile saline (n = 6) was infused into the CeA of rats. At specific time points after the infusions (2, 4, and 8 weeks), the rats (AAV2/5-GFP, n = 6; saline, n = 2; for each time point) were perfused and the brains were removed to determine GFP levels, transduction efficiency and tropism in the CeA.

Experiment 2: Overexpression of CRF in the CeA and brain reward function in nicotine-naïve and nicotine-withdrawing rats. The rats were trained on the ICSS procedure and when the thresholds were stable, AAV2/5-CRF (n = 23) or AAV2/5-CRF (n = 23) was infused bilaterally into the CeA. The ICSS threshold and response latencies were assessed daily throughout the experiment. Twenty-eight days after the vector infusions, the rats were prepared with 28-day minipumps that delivered saline (n = 11 per group) or nicotine (n = 12 per group, 3.16 mg kg⁻¹ per day, base). The nAChR antagonist mecamylamine (0.33–3.0 mg kg⁻¹, subcutaneous) was used to precipitate nicotine withdrawal. Mecamylamine was administered according to a Latin square design and the rats received the first dose 7 days after the pump implantations. There were at least 72 h between subsequent mecamylamine injections. The minipumps were removed at day 28 to study spontaneous withdrawal. The rats were tested in the ICSS procedure 6, 12, 24, 36, 48, 72, and 96 h after pump removal. When the experiment was completed, the brains were removed to assess CRF and CRF receptor levels. About half the animals from each group were used for western blotting (n = 6 per group) and the other half for RT–PCR (n = 4, saline groups; n = 5, nicotine groups).

**Figure 1.** AAV2/5-mediated expression of GFP in the CeA. The schematic figures (anterior posterior = − 2.30 mm from bregma) were adapted from Paxinos and Watson and illustrate the localization of the CeA (a, b). Immunofluorescent stainings revealed a high level of AAV2/5-mediated GFP protein expression in the CeA 2–8 weeks after the infusions (representative image taken 4 weeks post infusions, c). Scale bar, 200 μm. AAV, adeno-associated virus; BLA, basolateral amygdala; CeA, central nucleus of the amygdala; CPu, caudate putamen; GFP, green fluorescent protein; Pir, piriform cortex.
RESULTS

Experiment 1: Time course and tropism of AAV2/5-mediated GFP expression in CeA

Time course. The immunostainings showed that GFP was expressed in the CeA after the AAV2/5-GFP infusions (Figures 1a–c). GFP levels were similar 2, 4, and 8 weeks after the infusions (time: F2,14 = 1.301, NS; Supplementary Figures S1a and d). The saline-control animals did not express GFP.

Colocalization of GFP and NeuN in the CeA. To determine the AAV2/5-mediated transduction efficiency of neurons in the CeA, we examined the colocalization of GFP and the neuronal marker NeuN. Histological analysis showed that a great majority of the GFP-positive cells, 98%, were also NeuN-positive. This percentage was similar for all time points (time: F2,13 = 1.094, NS; Supplementary Figures S2a and b). The saline-control animals did not express GFP.

Colocalization of GFP, NeuN and CRF in the CeA. To investigate if AAV2/5 transduces CRF neurons in the CeA, we examined the colocalization of GFP, NeuN, and CRF. About 95% of the CRF-positive cells were also GFP and NeuN-positive and this percentage was similar for all time points (time: F2,9 = 0.595, NS; Supplementary Figures S2c and d).

Experiment 2: Overexpression of CRF in the CeA and brain reward function in nicotine-naïve and nicotine-withdrawing rats

Verification of AAV2/5-CRF vector in a primary neuronal cell culture. The transduction efficiency of the AAV2/5-CRF vector was investigated using a neuronal cell culture. The administration of AAV2/5-CRF increased CRF mRNA levels in primary neurons (1.4 × 10^11 genome copies, fourfold increase; 3.5 × 10^11 genome copies, sevenfold increase; F3,4 = 65.48, P < 0.001; Supplementary Figures S3a and b).

Overexpression of CRF in CeA and ICSS thresholds. The mean absolute ICSS thresholds and response latencies before the injection of the viral vectors are reported in Supplementary Table S1. There were no differences in ICSS thresholds or response latencies between the AAV2/5-CRF and AAV2/5-GFP group before the AAV2/5 injections. After the rats received the viral vectors, they were tested daily in the ICSS setup for 28 days. The analysis of variance indicated that the overexpression of CRF in the CeA did not affect the ICSS thresholds or the response latencies (Supplementary Figures S4a and b).

Effect of CRF overexpression in the CeA on precipitated nicotine withdrawal. There were no differences in ICSS thresholds or

Figure 2. Overexpression of CRF in the CeA diminishes the dysphoric-like state associated with nicotine withdrawal. Administration of mecamylamine (a) and minipump removal (c) elevated ICSS thresholds in the nicotine-treated rats (n = 11–12 per group). Withdrawal was diminished by the overexpression of CRF in the CeA. Mecamylamine (b) did not affect the latencies but removal of the nicotine pumps (d) led to a small increase in response latencies. Asterisks (**P < 0.01) indicate elevated ICSS thresholds compared with chronic-nicotine/vehicle group and all chronic-saline animals (a). Plus signs (++P < 0.01) indicate lower ICSS thresholds compared with GFP-nicotine animals that received the same dose of mecamylamine (a). Asterisks (**P < 0.01) indicate elevated ICSS thresholds compared with chronic-saline groups or increased latencies compared with the GFP-saline group (c, d). Plus signs (++P < 0.01) indicate lower ICSS thresholds compared with GFP-nicotine animals (c). Data expressed as mean ± s.e.m. CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; GFP, green fluorescent protein; ICSS, intracranial self-stimulation.
response latencies between the experimental groups (AAV2/5-CRF-nicotine, AAV2/5-CRF-saline, AAV2/5-GFP-nicotine and AAV2/5-GFP-saline) before the implantation of the minipumps or before the first mecamylamine injection (Supplementary Table S1). In addition, repeated administration of mecamylamine did not affect the baseline thresholds or latencies. Mecamylamine elevated the ICSS thresholds of the nicotine-treated rats, but did not affect the thresholds of the saline-treated rats (dose × pump: F_{3,126} = 46.23, P < 0.001, Figure 2a). Overexpression of CRF in the CeA diminished the mecamylamine-induced elevation in ICSS threshold in the nicotine-treated rats and did not affect the thresholds of the saline-treated rats (vector × pump: F_{1,42} = 4.42, P < 0.05; dose × pump × vector: F_{3,126} = 6.88, P < 0.001). Post hoc comparisons revealed that 1 and 3 mg kg\(^{-1}\) of mecamylamine elevated the ICSS thresholds of the nicotine-treated GFP rats. These doses also elevated the ICSS thresholds of the nicotine-treated rats that overexpressed CRF but to a lesser degree than those of the nicotine-treated GFP rats.

Mecamylamine increased the response latencies of the nicotine-treated rats and did not affect the response latencies of the saline-treated control rats (dose: F_{3,126} = 3.99, P < 0.01; pump: F_{1,42} = 4.83, P < 0.05, Figure 2b). The overexpression of CRF in the CeA did not affect the response latencies. Although mecamylamine increased the response latencies of the nicotine-treated rats, post hoc comparisons did not reveal significant differences between the groups.

Effect of CRF overexpression in the CeA on spontaneous nicotine withdrawal. There were no differences in ICSS thresholds or response latencies between the experimental groups before pump removal (Supplementary Table S1). Removal of the minipumps led to elevations in the ICSS thresholds in the nicotine-treated rats but not in the saline-treated control rats (time × pump: F_{6,252} = 13.66, P < 0.001, Figure 2c). The overexpression of CRF in the CeA diminished the elevations in the ICSS thresholds associated with nicotine withdrawal and did not affect the thresholds of the saline-treated control rats (time × vector: F_{6,252} = 3.21, P < 0.01; pump × vector: F_{1,42} = 8.65, P < 0.01; time × pump × vector: F_{6,252} = 3.69, P < 0.01). The post hoc analysis showed that the ICSS thresholds of the nicotine-withdrawing rats that overexpressed GFP were elevated from 6–48 h post pump removal. In contrast, the thresholds of the nicotine-withdrawing rats that overexpressed CRF were only slightly elevated at the 6-h time point. Furthermore, at the 6, 12, 24, and 48 h time points, the ICSS thresholds of the nicotine-withdrawing rats that overexpressed CRF were lower than those of the nicotine-withdrawing rats that overexpressed GFP. The removal of the nicotine pumps led to a small increase in the response latencies and the latencies gradually returned to baseline levels (time × pump: F_{6,252} = 4.30, P < 0.001, Figure 2d). The post hoc test indicated that at the 12-h time point, the latencies of the nicotine-withdrawing GFP rats were slightly longer than those of the saline-control rats that overexpressed GFP.

Gene transfer and CRF and CRF receptor levels in CeA. At the end of the ICSS experiments, the brains were removed and CRF protein and mRNA levels were assessed. Western blotting revealed a band of about 21 kDa, which corresponds to the molecular weight of the CRF precursor (prepro-CRF).\(^{32}\) The CRF levels in the CeA of animals that had received AAV2/5-CRF were 40% higher than those in animals that had received AAV2/5-GFP (t_{122} = 5.56, P < 0.001, Figures 3a and b). The CRF mRNA were also 40% higher in the AAV2/5-CRF rats than in the AAV2/5-GFP rats (t_{122} = 2.22, P < 0.05, Figure 3a). Furthermore, the RT–PCR analysis showed that the overexpression of CRF led to a decrease in CRF\(_1\) receptor mRNA levels (t_{120} = 2.42, P < 0.05) and increase in CRF\(_2\) receptor mRNA levels (t_{120} = 1.13, P < 0.05, Figure 3c).

A correlation analysis was conducted to further investigate the relationship between ICSS thresholds during nicotine withdrawal and CRF\(_1\) and CRF\(_2\) receptor levels in the CeA. There was a positive correlation between CRF\(_1\) mRNA levels and ICSS thresholds after the administration of 1 mg kg\(^{-1}\) (r = 0.550, P < 0.05) and 3 mg kg\(^{-1}\) of mecamylamine (r = 0.625, P < 0.05) to the nicotine dependent rats, and 6 h after the removal of the nicotine pumps (r = 0.806, P < 0.01, Supplementary Table S2). Furthermore, there was a negative correlation between CRF\(_2\) mRNA levels and ICSS thresholds after the administration of 3 mg kg\(^{-1}\) of mecamylamine (r = 0.592, P < 0.05). There were no significant correlations with lower doses or later time points. This correlations analysis suggests that animals with high CRF\(_1\) mRNA expression levels and low CRF\(_2\) mRNA expression levels in the CeA are most likely to have a large increase in the ICSS thresholds during nicotine withdrawal.
DISCUSSION

The present studies investigated the effect of AAV2/5-mediated overexpression of CRF in the CeA on precipitated and spontaneous nicotine withdrawal in rats. The overexpression of CRF diminished the elevations in ICSS thresholds associated with precipitated and spontaneous nicotine withdrawal. The overexpression of CRF also led to a decrease in CRF1, and increase in CRF2 receptor mRNA levels. This suggests that the overexpression of CRF in the CeA diminishes nicotine withdrawal at least partly by decreasing CRF1 receptor mRNA levels and increasing CRF2 receptor mRNA levels. These data suggest that the overexpression of CRF leads to neuroadaptive changes in CRF receptor synthesis to prevent the harmful effects of a prolonged increase in CRF levels.17

The transduction efficiencies of AAV vectors depend on the pseudotype of the vector and the brain region.33,34 The AAV2/5 vector transduces a variety of brain areas with a high efficiency.34 However, transduction efficiency, tropism and time course of transgene expression in the CeA was not known. Therefore, we assessed AAV2/5-mediated GFP expression in the CeA at various time points. There was a strong increase in GFP levels in the CeA, 2, 4 and 8 weeks after the AAV2/5-GFP infusions. GFP levels were slightly higher at the 4-week time point than at the 2-week time point but this difference was not significant. This is in line with a study that showed that AAV2/5-mediated GFP expression in the striatum is similar 2 and 4 weeks after the infusions.35 Although we did not investigate GFP expression at very early time points, it has been reported that AAV2/5-mediated GFP expression levels in the striatum gradually increase and asymptote by 2 weeks.35 We then investigated cell tropism of the AAV2/5 vector. The confocal analysis showed that 98–99% of the GFP-positive cells in the CeA expressed the neuronal marker NeuN. These results are similar to a previous study that evaluated cell tropism of AAV2/5.35 In that study, all the brain cells that were transduced by AAV2/5-GFP expressed NeuN and none of the cells expressed the astrocyte-specific marker GFAP. Finally, we determined if AAV2/5 transduces CRF cells in the amygdala. The viral vector, AAV2/5, transduced about 95% of the CRF/NeuN-positive cells that were evaluated. This indicates that AAV2/5 is an excellent vector to overexpress CRF in neurons in the CeA.

The present study showed that the overexpression of CRF in the CeA diminishes the dysphoric-like state associated with precipitated and spontaneous nicotine withdrawal in rats. Initially it seems counterintuitive that an increased expression of this stress peptide diminishes the negative mood state associated with nicotine withdrawal. The present finding seems to contradict previous studies that showed that the release of CRF contributes to the dysphoric-like state associated with nicotine withdrawal.24,31 Pharmacological studies also indicate that the acute administration of CRF into the brain leads to a negative mood state as indicated by elevations in the ICSS thresholds and conditioned place aversion.26,37 The acute administration of CRF also increases anxiety-like behavior.38,39 Our finding of this can be partly explained by a recent study that suggests that the overexpression of CRF in rats leads to the development of tolerance to the effects of CRF.25 It was shown that prolonged lentiviral-mediated overexpression of CRF in the CeA leads to a decrease in anxiety-like behavior in the light-dark box test and this effect was most notable after exposure to a stressor.25 Although the development of tolerance to the effects of CRF has not been widely investigated, other studies have shown that animals develop tolerance to the effects of neuropeptides such as orphanin FQ and melanin-concentrating hormone.40,41 It is possible that the development of tolerance to the dysphoric-like effects of CRF is mediated by CRF-induced changes in CRF receptor levels. In the present study, we showed that the overexpression of CRF leads to a decrease in CRF1 and increase in CRF2 receptor mRNA levels. This observation is in line with the other studies that investigated the effects of stress or CRF overexpression on CRF receptor levels. Transgenic mice that overexpress CRF have lower CRF1 receptor mRNA levels and higher CRF2 receptor mRNA levels than wild-type control mice.42 Furthermore, social stress leads to the internalization of CRF1 receptors and the recruitment of CRF2 receptors to the plasma membrane.43 These studies suggest that a chronic activation of stress systems, either by the overexpression of CRF or exposure to stressors, leads to a decrease in the expression of CRF receptors and an increase in CRF2 receptors. The activation of CRF1 receptors contributes to negative mood states and CRF2 receptor activation has opposite effects and counteracts the effects of CRF1 receptor activation.44–46 The notion that CRF2 receptor activation has antidepressant-like effects is supported by studies that show that the CRF2 receptor agonists urocortin 2 and 3 have antidepressant-like effects in the forced swim test.47 Furthermore, CRF2 receptor knockout mice have a depressive-like phenotype in the forced swim test and tail suspension test.48,49 It should be noted that although there is strong evidence that CRF2 receptor activation has antidepressant-like effects, some studies suggest that CRF2 receptor blockade or knockdown prevents dysphoric-like states.48,49 One potential limitation of our study was that the effect of CRF overexpression on CRF receptor levels was investigated in rats that had undergone several experimental procedures. Although the brains were collected at least several days after the reward thresholds had returned to baseline levels, it cannot be completely ruled out that some of the experimental procedures affected CRF receptor levels.

The present studies show that prolonged AAV-mediated overexpression of CRF in the CeA diminishes the dysphoric-like state associated with precipitated and spontaneous nicotine withdrawal. Furthermore, chronic overexpression of CRF led to a decrease in CRF1 receptor gene expression and an increase in CRF2 receptor gene expression. Previous studies showed that the negative mood state associated with nicotine withdrawal is at least partly mediated by an increase in CRF release in the CeA.21,24,31 Taken together, these studies suggest that chronic AAV-mediated overexpression of CRF in the CeA diminishes the nicotine withdrawal-induced dysphoric-like state, possibly by inducing adaptations that blunt the effects of the increase in CRF in the CeA during nicotine withdrawal. These studies point to a pivotal role for CRF signaling in the CeA in tobacco addiction. Gene therapy or other treatments that diminish the effects of CRF in the CeA during a smoking cessation attempt might prevent dysphoria and improve relapse rates.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was funded by a National Institute on Drug Abuse grant (DA023575) and a Flight Attendant Medical Research Institute Young Clinical Scientist Award (052312) to AWB.

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