Masked Smoking-Related Images Modulate Brain Activity in Smokers

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Abstract: The questions of whether and how indiscriminate drug-related stimuli could influence drug-users are important to our understanding of addictive behavior, but the answers are still inconclusive. In the present preliminary functional magnetic resonance imaging study using a backward masking paradigm, the effect of indiscriminate smoking-related stimuli on 10 smokers and 10 nonsmokers was examined. The BOLD response showed a significant reduction ($P = 0.001$) in the right amygdala of smokers when they viewed but did not perceive masked smoking-related stimuli, while no significant differences were found in the nonsmoker group. More voxels in anterior cingulate cortex were negatively correlated with the amygdala during the masked smoking-related picture condition in smokers but not in nonsmokers, whereas more positively correlated voxels were observed during the masked neutral condition. The BOLD response in drug-users indicates the amygdala responds to drug-related stimuli that are below the perceptual threshold. The functional connectivity data suggest a functional interaction between the amygdala and the anterior cingulate cortex when drug users view 33ms back-masked drug-related stimuli. This observation suggests that the amygdala plays an important role in the indiscriminate drug-related cue process. *Hum Brain Mapp 30:896–907, 2009.*   © 2008 Wiley-Liss, Inc.

Key words: unawareness; smoking-related cue; functional magnetic resonance imaging (fMRI); amygdala; addiction

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INTRODUCTION

Nicotine addiction is the leading preventable cause of death in developed countries [Graul and Prous, 2005]. Millions of nicotine users are willing to stop but few of them are able to do so [Graul and Prous, 2005] because addiction is a compulsive pattern of drug-related behaviors including drug-seeking and drug-taking [Everitt and Robbins, 2005; Koob and Le, 2001; Robinson and Berridge, 1993, 2003; Tiffany, 1990; Tiffany and Conklin, 2000; Volkow and Li, 2004]. The reason for the compulsivity of addictive behaviors and the difficulty for drug-users to stop using drugs are central questions in addiction [Edwards et al., 1981; Robinson and Berridge, 2003; Volkow and Li, 2005].

Behaviors related to drug seeking or taking tend to occur relatively fast, often without awareness [Robinson and Berridge, 2003; Tiffany, 1990]. Therefore, the unawareness process is the important addiction characteristics [Tiffany, 1990] and involved in smoking initiation [Kremers et al., 2004]. However, to date, the effects of unawareness processing of drug-related visual input remain controversial. For example, it was found that in abstinent crack cocaine-dependent men, visual scanning of the preattentive (indiscriminate) cocaine-related visual cue was modulated by their cocaine craving scores [Rosse et al., 1997]. In another study, Ingjaldsson et al., [2003] also reported that alcoholics showed heart rate deceleration after exposure to masked and invisible alcohol-related pictures. In comparison, other studies reported contradictory results [Bradley et al., 2004; Franken et al., 2000; Mogg and Bradley, 2002], including the lack of attentional bias in smokers for indiscriminate smoking-related pictures followed by a visual probe [Bradley et al., 2004] and the absence of an indiscriminate attentional effect for drug-related words in a modified Stroop task, in which the drug-related words were presented instead of the color words [Franken et al., 2000; Mogg and Bradley, 2002]. This discrepancy may be attributed mainly to the difference in measurements used in the above studies. To help resolve this discrepancy, it is important to study the potential cortical and subcortical responses to the masked drug-related visual input.

To date, no published neuroimaging studies have specifically examined the effect of drug-related stimulus in the absence of input awareness. Existing studies of the neural basis of processing indiscriminate visual stimuli are limited exclusively to emotional stimuli [Pessoa, 2005]. Among the structures identified, the amygdala is perhaps the most important brain area in the unawareness processing of emotional information [Etkin et al., 2004; Killgore and Yurgelun-Todd, 2004; LeDoux, 1996, 2000; Whalen et al., 1998, 2004] along with the anterior cingulate cortex (ACC) [David et al., 2005; Gallagher and Frith, 2003; Killgore and Yurgelun-Todd, 2004; LeDoux, 2000]. Additionally, the amygdala and the ACC are also the most commonly reported loci of activation induced by drug-related cues in brain-imaging studies [Brody et al., 2004;

David et al., 2005; Franklin et al., 2007; Lee et al., 2005; Lim et al., 2005; McBride et al., 2006; Wilson et al., 2004, 2005]. For example, Due et al., [2002] reported that neural substrates are modulated by explicit visual smoking-related cues in smokers. They observed stronger BOLD signals in several brain areas (e.g., amygdala, ACC, DLPFC, etc.) when smokers were exposed to smoking-related images than when they were exposed to neutral images and that these conscious smoking-related images also affected the smokers' craving levels [Due et al., 2002].

In this study, we investigated the neural processing of indiscriminate smoking-related stimulus with functional magnetic resonance imaging (fMRI). A traditional backward masking paradigm was used to render the stimulus indiscriminate, and an fMRI paradigm was used to ascertain which brain locations generated significant indiscriminate smoking-related stimuli activation in a smoker group and a control (nonsmoker) group. On the basis of the above discussion, we hypothesize that the indiscriminate smoking-related cue would influence the BOLD response in some brain areas (e.g., the amygdala).

Additionally, there is evidence [Bokde et al., 2006; Buchel et al., 1999; Williams et al., 2006a] showing that the BOLD response and the connectivity may reveal different aspects of the brain mechanisms of a cognitive function. Functional connectivity between brain regions has been defined as the temporal correlation between spatially remote neurophysiological events [Friston et al., 1993]. Previous research [Jacobsen et al., 2004, 2007] indicates that nicotine-abuse may be dependent on a distributed neuronal network consisting of cortical and limbic regions rather than on the activity of a discrete brain region. Therefore, functional brain abnormalities in smokers may be present in the functional connectivity between brain regions as well as within discrete brain regions. Particularly, ACC is found to have direct neural connections to the amygdala [LeDoux, 1996, 2000]. It is predicted that, in this study, the correlation between different regions (e.g., the amygdala and the ACC) for the assessment of functional connectivity in smokers will be influenced by indiscriminate smoking-related cues.

METHODS AND MATERIALS

Participants

Twenty right-handed healthy adults participated in this study after providing informed consent as approved by the Anhui Medical University review board. Half of the participants (mean age: 25.10 ± 1.07, range: 18–29) were smokers who had a history of smoking for at least 1.5 years (mean: 4.85 ± 1.02 years, range: 1.5–10) and consumed >10 cigarettes/day (consistent with the criterion in Bradley's [2003] study). The other half were nonsmokers (mean age: 23.80 ± 0.81, range: 21–28) who never smoked during their lifetime. Groups did not significantly differ on age ($t = 0.967$, ns). None of the participants had a history of any neuropsychiatric disorders or other drug depend-
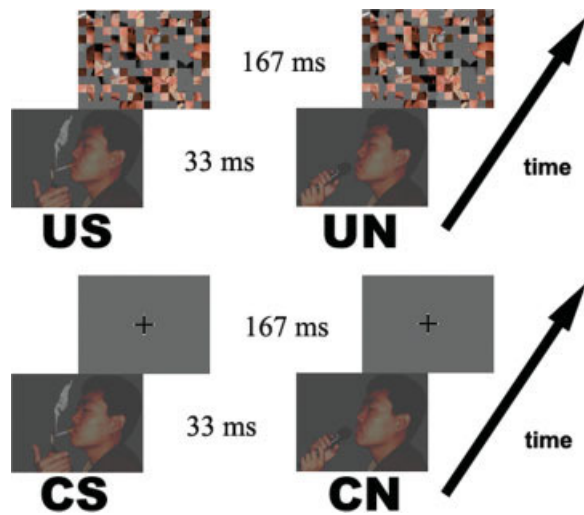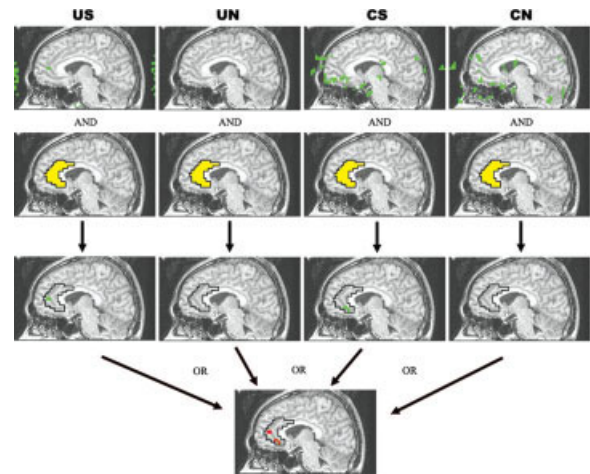
Figure 1.

Sample stimuli and experimental parameters in the four tasks (US/UN/CS/CN). There were two kinds of stimulus pictures, i.e., smoking-related and neutral. One representative pair was showed above.

Figure 2.

The definition of ROI. There is a schematic diagram for definition of ROI of the right ACC in one representative participant. The green area is the activation map (after clustered) in each task (US/UN/CS/CN). The yellow area is the right ACC based on the anatomical structure and stereotaxic coordinates [Talairach and Tournoux, 1988]. The red area is the ROI of the right ACC in this participant. The definition of the ROIs in the OTC, OVC, DLPFC, and IFG are same as that of the right ACC.

ence. Only male participants were selected as gender effect was not a focus of this study. Smokers also went through the Fagerstrom test for nicotine dependence [Heatherton et al., 1991; Moolchan et al., 2002] and scored an average of 5.00 (SD:1.63, range:3–7). All participants confirmed verbally that they abstained from nicotine, alcohol, coffee, and tea starting from the preceding midnight. The participant's abstinent status was monitored by experimenters for 2 h prior to scanning.

Paradigm and Procedure

On the basis of our pilot experiments and previous studies of unconscious drug-related cues [Bradley et al., 2003; Franken et al., 2000; Mogg and Bradley, 2002] and emotional cues [Killgore and Yurgelun-Todd, 2004; Whalen et al., 1998], the signal associated with the indiscriminate stimulus was expected to be very small. To ensure that we could detect this effect, the block design was used as it provides robust results [Loubinoux et al., 2001; Machielsen et al., 2000; Rombouts et al., 1997, 1998], a relatively high statistical power [Friston et al., 1999] and a relatively large BOLD signal change related to baseline [Buxton et al., 1998]. Additionally, in our pilot experiment, some participants reported they were able to see the masked stimuli, especially when the indiscriminate condition was presented following the discriminate condition. Therefore, in this study, all indiscriminate scanning runs were conducted before the discriminate scanning runs.

All participants attended one scan session including six passive viewing scanning runs for the indiscriminate condition. Because the signal associated with the subliminal stim-

ulus was expected to be very small, we tried to collect subliminal data as much as possible during the limited scanner time. Therefore, only fourteen of them (seven smokers and seven nonsmokers) completed another two passive viewing scanning runs for the discriminate condition following the indiscriminate scanning runs. In each scanning run, there were 13 blocks consisting of six stimulation blocks and seven baseline blocks. The stimulation blocks were separated by the baseline blocks, in which a fixation cross ($1° × 1°$ visual angle) was displayed on the screen. Each block lasted 20 s, except for the first and the last baseline blocks, which were 10-s long for equaling the number of time points between the baseline and the cognitive task.

In indiscriminate scanning runs (i.e., the indiscriminate condition), there were two categories of stimulation blocks: indiscriminate (unconscious) smoking-related (US, three blocks) and indiscriminate (unconscious) neutral (UN, three blocks). The order of the US and UN blocks was counterbalanced across the participants. Each stimulation (i.e., US or UN) block consisted of 10 trials. Each trial in the indiscriminate scanning runs was a traditional backward masking presentation trial as used in previous studies [Killgore and Yurgelun-Todd, 2004; Morris et al., 1998, 1999; Sheline et al., 2001; Whalen et al., 1998]. The trial started with a 33-ms presentation of a stimulus picture, followed by a masking picture displayed for 167 ms and an 1,800-ms fixation cross (see Fig. 1). There were 10 smoking-related stimulus pictures ($6.5° × 5°$ in visual angle) showing a man smoking a cigarette with different poses.

Ten paired neutral pictures showed the same man holding a microphone with the same pose as that of the corresponding smoking-related pictures (see Fig. 1). The background in all pictures was discarded to decrease the interference from other information. The mask picture was the result of randomization of pieces in the stimulus pictures such that it did not have any perceivable object but shared the color and texture of the stimulus pictures (same to the previous studies [Ingjaldsson et al., 2003; Ohman and Soares, 1993]). The stimuli were presented on a screen through a projector controlled by a computer.

According to the previous study [Williams et al., 2004], stimulus detection remains possible and masking interferes only with the subsequent ability to discriminate it at the 33 ms presentation of stimuli. Therefore, the indiscriminate condition in this study is at the discrimination but not the detection level [Williams et al., 2004]. The criterion of "indiscriminate" in this study is determined by the ability of the participants to observe the key items (i.e., cigarette, microphone).

The trials in the discriminate condition were similar to those in the indiscriminate condition. The only difference was that the fixation was displayed instead of the masking picture after the task picture so that the participants were able to perceive the stimulus consciously (see Fig. 1). Similar to the indiscriminate condition, there were two types of task blocks: discriminate (conscious) smoking-related (CS) and discriminate (conscious) neutral (CN).

Data Acquisition

Images were obtained with a GE 1.5T MR scanner (GE Medical Systems, Milwaukee, WI). A circularly polarized head coil was used, with foam padding to restrict head motion. Functional images were acquired with a T2*-weighted echo-planar imaging sequence (TE = 45ms, TR = 2s, FOV = 24 cm) with 22 axial slices (no slice gap, one voxel: $3.75 \times 3.75 \times 4$ mm^3). Corresponding high-resolution T1-weighted spin-echo (for anatomical overlay) images and three-dimensional gradient-echo (for stereotaxic transformation) images were also collected. Each functional run lasted 4 min 8 s (124 images/slice). The first four time points in each run were discarded to account for the approach to steady state in the BOLD signal.

Data Analysis

The imaging data were analyzed with analysis of functional neuroimages (AFNI), [Cox, 1996]. As detailed below, data analysis included the BOLD response and the functional connectivity.

Analysis of BOLD response

Activation map generation. The raw data were motion corrected and normalized. Then, the six functional scanning runs for the indiscriminate condition and the two scanning runs for the discriminate condition were con-

catenated, respectively, to obtain a combined data set for each condition. Subsequently, correlation analysis was performed on these two data sets based on the contrasts between the tasks and the baseline to generate corresponding activation maps (threshold: $P < 0.05$) for each participant. A spatial cluster size threshold of four connected voxels was applied to the activation maps to eliminate isolated sporadic active voxels. With the spatial clustering, the false positive level, corrected for multiple comparison in the entire imaged volume, was <0.001 (Monte Carlo simulation conducted with AFNI). An example of the activation map in one representative participant is showed in the first row in Figure 2.

Regions of interest definition. Several brain regions were selected as regions of interest (ROI) if there are one or more activation clusters within an anatomically delineated area in at least eight out of 10 participants and one or more activation clusters within the same anatomical area in every participant during at least one task. These areas included bilateral occipital/temporal cortex (OTC, BA37), bilateral occipital visual cortex (OVC, BA17/18/19), bilateral dorsolateral prefrontal cortex (DLPFC, BA9/46), bilateral inferior frontal gyrus (IFG, BA44/45) and right ACC (BA24/32). A ROI is defined for each of these areas in each participant based on the union of activations for any of the tasks (US\UN\CS\CN) within an anatomically delineated area (see Fig. 2). The size of these ROIs averages 23–131 voxels.

In addition to the areas selected based on activation, bilateral amygdala, bilateral caudate and bilateral pulvinar were also considered because they have been suggested to be involved in unconscious processing of emotional and drug-related visual cues [Etkin et al., 2004; Killgore and Yurgelun-Todd, 2004; LeDoux, 1996, 2000; Morris et al., 1999; Wilson et al., 2004]. Since these ROIs did not consistently show significant activation in the activation maps, they were defined based on anatomical structures and stereotaxic coordinates [Talairach and Tournoux, 1988]. The ROIs in these regions contained 29 voxels for amygdala (1.6–1.7 cm^3, which is consistent with previous studies [Brierley et al., 2002; Robinson et al., 2004]; see details in the next paragraph and Fig. 4A), 47 voxels for pulvinar, and 96 voxels for caudate. No significant differences were found between the smoker group and the nonsmoker group in the volume of these ROIs [the amygdala (left: $t = 0.107$, ns; right: $t = 0.788$, ns), the pulvinar (left: $t = 0.551$, ns; right: $t = 0.973$, ns) and the caudate (left: $t = 0.081$, ns; $t = 0.788$, ns)].

All three planes were used to determine the boundaries of the amygdala following the previous studies [Sheline et al., 2001; Gur et al., 2000, 2004]. Visualized in the coronal section, the anterior boundary of the amygdala was the first section in which the white matter connecting the frontal and temporal lobes became continuous. The posterior boundary was drawn adjacent to the anterior boundary of the hippocampus. The dorsal boundary was determined first by the coronal slice cutting through the most inferior-

anterior point of the temporal horn of the lateral ventricle (chosen from the sagittal plane). Ventrally, visualized in the sagittal section, the amygdala was bounded by a horizontal line connecting to the ventral edge of the hippocampus. Medially, seen in the coronal section, the amygdala was bounded by subarachnoid space. Laterally, seen in the coronal section, the amygdala was bounded by white matter.

Characterization of BOLD response. The time courses of the BOLD signal were segmented into blocks for each task and the first time point of each block was removed to minimize the effects of hemodynamic delay. The time courses for each task were then averaged across all the voxels in each ROI. The amplitude of BOLD response was obtained by the average BOLD signal within the duration of each task (US/UN/CS/CN).

Sub-group analysis. To test whether the BOLD response difference between the US and the UN conditions (i.e., UN minus US) in the smoker group is related to the addiction level, a correlation analysis between the number of cigarettes smoked per day and the BOLD response to the indiscriminate tasks (UN minus US) was performed in the ROIs where the BOLD difference (UN vs. US) was significant in smokers.

Analysis of functional connectivity

Preprocessing. For the purpose of performing functional connectivity analysis, four preprocessing steps (see Fig. 3) were applied to the raw functional data, following the pro-

cedure described in previous studies [Hashimoto and Sakai, 2004; Homae et al., 2003]. Specifically, the preprocessing steps include: (a) correction of temporal shifts between slices, (b) motion correction and normalization, (c) separation of fMRI time courses into blocks for each task with the first time point removed to minimize the effect of the hemodynamic response delay, and (d) construction of task-specific (US/UN/CS/CN) time courses by concatenating the time courses of individual task blocks in each run and averaging across the repeated runs. Thus, each of the resultant task-specific time courses contained 27 time points.

Functional connectivity calculation. The right amygdala, the only area exhibited significant differences in BOLD response between the US and the UN in smokers, was selected as the seed ROI in this study. The other relevant brain areas (i.e., the bilateral OTC, OVC, DLPFC, IFG, caudate, pulvinar, and the right ACC) in the BOLD response analysis were selected as the target ROIs. Functional connectivity between the seed ROI (the right amygdala) and the target ROIs was calculated. Functional connectivity was evaluated by examining the volume of the voxels with significant positive or negative correlation coefficient (threshold: $r > 0.5$ or $r < -0.5$, $P < 0.0093$, uncorrected) within each target ROI. The analysis protocol for functional connectivity analysis in this study was same to that in previous published studies [Homae et al., 2003; Stein et al., 2000].

RESULTS

Debriefing Participants

After entering the scanner but before starting the fMRI data collection, the participants were examined individually to assess the effect of backward masking using the same set of pictures later used in the fMRI runs. To eliminate the subjective bias as much as possible, the participants were required not only to report whether they could see the pictures or not but also to describe what they saw in detail. Once the word "cigarette" or "microphone" appeared in the participant's report, the luminance and the contrast of the stimulus pictures were reduced until the participant did not report any meaningful scenes. Two participants (one smoker and one nonsmoker) were found to need such reduction. The reduction was applied in both indiscriminate and discriminate conditions. After the fMRI runs were completed, the participants were examined in the same manner again to ensure the effectiveness of the masking and to make sure that the masked stimulus remained invisible. All participants reported that none of pictures with the mask but all of pictures without the mask were perceived. Similar to Morris' [1998] study, some participants reported awareness of flickering occurring during the unconscious presentations, but did not report the perception of any meaningful scenes, especially the images of the cigarette or the microphone.
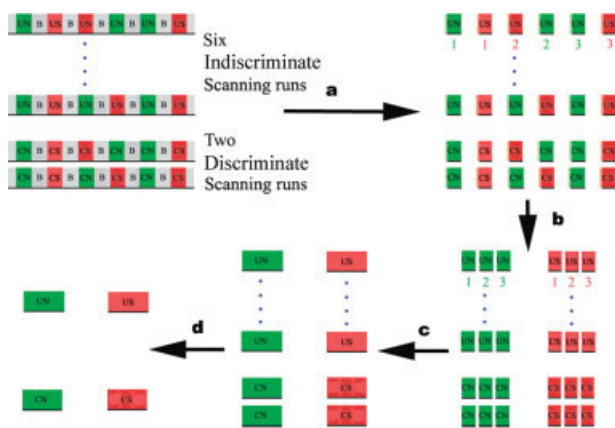
Figure 3.

The preprocess of functional connectivity analysis. (**a**) The time courses of BOLD signals were segmented into blocks for each task (smoking-related and neutral, respectively), with the first time point removed to minimize the effect of hemodynamic changes from its preceding task block. (**b,c**) Task-specific (US, UN, CS, and CN) time courses were constructed by merging all the time courses of individual task blocks into one scan. (**d**) The resultant task-specific time courses were averaged across the scans, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. The BOLD response of each ROI (%), threshold $P < 0.05$

	Smoker				Nonsmokers				ANOVA	
	Smoking -related	Neutral	t	P	Smoking -related	Neutral	t	P	F	P
Indiscriminate										
Left										
Amygdala	0.08	0.05	0.386	—	0.02	−0.02	1.347	—	0.046	—
Pulvinar	0.04	−0.04	1.985	—	−0.01	0.01	0.626	—	3.778	—
DLPFC	0.21	0.05	1.689	—	0.17	0.07	1.148	—	0.293	—
IFG	0.40	−0.01	1.247	—	0.13	0.12	0.170	—	1.353	—
OVC	0.35	0.31	0.455	—	0.37	0.32	0.790	—	0.004	—
OTC	0.25	0.019	0.575	—	0.28	0.31	0.302	—	0.403	—
Caudate	0.00	0.04	0.898	—	0.02	−0.01	1.087	—	1.897	—
Right										
Amygdala	−0.11	0.06	4.506	0.001	−0.02	0.01	0.609	—	7.135	0.016
Pulvinar	0.00	−0.01	0.122	—	0.00	−0.04	0.901	—	0.240	—
ACC	0.20	−0.15	1.112	—	0.13	0.10	0.443	—	0.972	—
DLPFC	0.12	0.11	0.050	—	0.54	0.11	1.087	—	0.959	—
IFG	0.11	−0.00	0.993	—	0.28	−0.00	2.067	—	1.007	—
OVC	0.31	0.36	0.555	—	0.41	0.36	1.032	—	0.975	—
OTC	0.23	0.29	0.708	—	0.34	0.25	1.530	—	2.073	—
Caudate	0.02	0.00	0.515	—	0.01	0.02	0.290	—	0.346	—
Discriminate								—		
Left								—		
Amygdala	0.13	0.03	0.416	—	−0.06	0.09	1.293	—	0.884	—
Pulvinar	−0.05	0.04	0.886	—	0.07	−0.01	0.995	—	1.735	—
DLPFC	0.78	−0.02	4.698	0.005	0.15	0.50	1.133	—	10.752	0.008
IFG	0.40	0.76	0.454	—	0.71	0.29	1.857	—	0.896	—
OVC	0.65	0.53	0.389	—	0.61	0.34	1.622	—	0.198	—
OTC	0.70	0.72	0.079	—	0.84	0.74	1.018	—	0.177	—
Caudate	0.16	−0.06	2.959	0.025	0.04	−0.02	1.294	—	2.892	—
Right								—		
Amygdala	0.40	−0.28	3.517	0.013	−0.01	−0.02	0.116	—	8.177	0.014
Pulvinar	−0.03	0.06	0.553	—	0.04	0.03	0.322	—	0.392	—
ACC	−0.10	1.13	0.973	—	0.28	0.25	1.352	—	0.940	—
DLPFC	0.64	0.018	1.466	—	0.55	0.27	1.272	—	0.227	—
IFG	0.92	0.46	2.110	—	0.43	0.68	0.822	—	3.528	—
OVC	0.66	0.60	0.248	—	0.71	0.44	2.379	—	0.514	—
OTC	1.31	0.09	2.746	0.033	0.74	0.59	1.001	—	5.206	0.042
Caudate	0.05	−0.02	1.676	—	0.06	−0.04	1.602	—	0.105	—

BOLD Responses

Because of the different number scanning runs and fixed order between the discriminate and the indiscriminate conditions and only seven smokers and seven nonsmokers were collected the discriminate data, we focused on the difference of the patterns of BOLD responses (i.e., the result of the smoking-related task vs. the neutral task) between the discriminate and the indiscriminate conditions instead of the direct comparison between these two conditions.

Indiscriminate Condition

The repeated-measures analysis of variance with a between-subjects factor comparing the addiction group (smoker and nonsmoker) and a within-subjects factor comparing the stimulus (smoking-related and neutral) revealed there was a significant interaction (threshold: $P < 0.05$) between the addiction group and the stimulus type in the right amygdala [$F(1,18) = 7.135$, $P = 0.016$; no main effect on the addiction group, $F(1,18) = 0.457$, ns; and significant

main effect on the stimulus, $F(1,18) = 12.616$, $P = 0.002$]. A planned contrast t-test (paired t-test, threshold $P < 0.05$) in the smoker group in this ROI ($t = 4.506$, $P = 0.001$) showed that the BOLD response during the presentation of indiscriminate smoking-related pictures was lower than during the presentation of indiscriminate neutral pictures, while no significant difference ($t = 0.609$, ns) was found in the nonsmoker group (Table I, Fig. 4).

In particular, the significant decrease of BOLD response in the UN than in the US in the right amygdala was influenced significantly by the number of cigarettes consumed by the smokers per day ($r = 0.858$, $P = 0.001$, Fig. 4). This result suggests that the BOLD response difference between the US and the UN may be functionally related to the level of nicotine use.

Discriminate Condition

The repeated-measures analysis of variance revealed there was a significant interaction between the addiction group and the stimulus in the right amygdala [$F(1,12) = 8.177$,
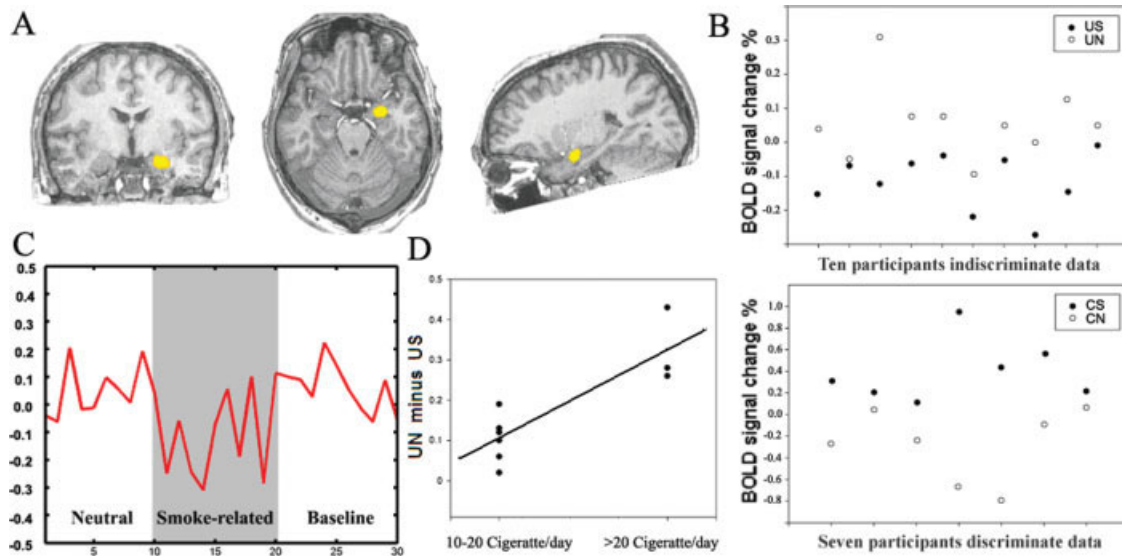
Figure 4.

Position of the amygdala and the BOLD responses under different tasks. (**A**) The boundaries for the right amygdala in this study. The boundaries for the right amygdala are displayed in Talairach space in this figure only for presentation; however, we calculated the BOLD response in individual original space. (**B**) A graphical display of individual BOLD response of the right amygdala in the US, UN, CS, and the CN in smokers. All smokers showed the BOLD response of the US was significantly lower than that of the UN, while the BOLD response of the CS was significantly higher than that of the CN. (**C**) The averaged time-course of the right amygdala from the smokers in indiscriminate tasks. (**D**) A graphical display of the difference in BOLD response between the UN and the US in the right amygdala. The smokers who consume over 20 cigarettes per day showed larger difference BOLD response between the UN and the US than the persons who smoke 10–20 cigarettes per day.

$P = 0.014$; no main effect on the addiction group, $F(1,12) = 1.251$, ns; and significant main effect on the stimulus, $F(1,12) = 8.760$, $P = 0.012$], the left DLPFC [$F(1,12) = 10.752$, $P = 0.008$; no main effect on the addiction group, $F(1,12) = 0.125$, ns; and no main effect on the stimulus, $F(1,12) = 1.693$, ns] and the right OTC [$F(1,12) = 5.206$, $P = 0.042$; no main effect on the addiction group, $F(1,12) = 0.074$, ns; and significant main effect on the stimulus, $F(1,12) = 8.535$, $P = 0.013$] in the discriminate conditions. A planned contrast t-test (paired t-test) in the smoker group in these ROIs [the right amygdala ($t = 3.224$, $P = 0.018$), the left DLFPC ($t = 4.698$, $P = 0.005$), the right OTC ($t = 2.746$, $P = 0.033$)] showed that the BOLD response during the presentation of discriminate smoking-related pictures was higher than during the presentation of neutral pictures, while no significant difference was found in the nonsmoker group [the right amygdala ($t = 0.116$, ns), the left DLFPC ($t = 1.583$, ns), the right OTC ($t = 1.001$, ns)] (Table I, Fig. 4).

Functional Connectivity

Indiscriminate condition

The repeated-measures analysis of variance with a between-participants factor comparing the addiction group (smoker and nonsmoker), a within-participants factor comparing the stimulus (smoking-related and neutral) and a
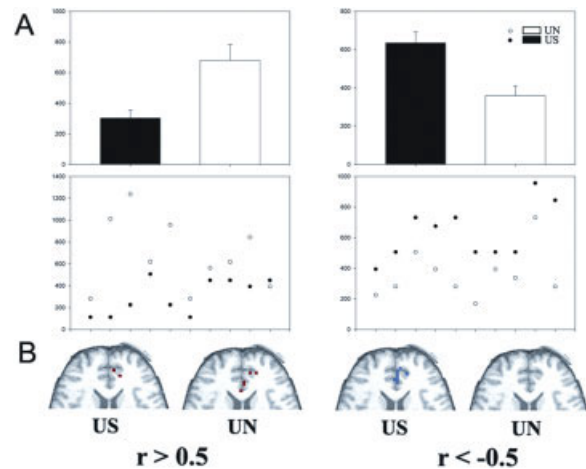
Figure 5.

Functional connectivity result between the right ACC and the right amygdale. (**A**) A graphical display of the averaged (up row) and individual (below row) volume of the voxels in which r value exceeded the threshold in the right ACC. The volume of the positively correlated voxels ($r > 0.5$) of the US was less than the UN (left), while the volume of the negatively correlated voxels ($r < -0.5$) of the US was more than the UN (right). Bars represent standard errors. (**B**) The significant positively ($r > 0.5$, red voxels) and negatively ($r < -0.5$, blue voxels) correlated voxels in the right ACC in one smoker.

TABLE II. Mean volume (mm^3) of the voxels in each ROI exceeding a significance criterion ($r > 0.5$ or < -0.5) in functional connectivity analysis

	Unconscious				Conscious				Unconscious				Conscious			
	$r > 0.5$		$r < -0.5$		$r > 0.5$		$r < -0.5$		$r > 0.5$		$r < -0.5$		$r > 0.5$		$r < -0.5$	
ROI	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N
Left																
Pulvinar	84	141	39	73	64	64	88	96	79	68	62	56	32	129	40	48
IFG	383	433	287	287	410	707	273	426	377	366	422	411	297	321	329	354
Caudate	146	231	129	124	257	394	177	121	191	219	163	180	145	169	177	145
OVC	917	1266	872	1103	1213	972	1069	1117	1142	1035	984	1204	956	1037	1004	1109
OTC	225	315	191	186	257	362	104	225	293	253	236	236	233	193	153	233
DLPFC	294	188	150	294	309	309	178	366	406	294	406	337	291	413	366	291
Right																
ACC	304	681	636	360	611	1069	370	579	551	579	495	563	281	378	402	410
Pulvinar	84	113	96	113	129	96	104	137	101	96	51	90	24	96	104	80
IFG	388	461	383	366	771	812	394	329	467	422	388	354	346	321	338	378
Caudate	152	191	113	158	217	305	177	113	152	158	197	203	137	177	96	193
OVC	1182	1496	928	1305	1021	1655	996	1310	1249	962	883	1176	1029	844	852	1013
OTC	186	298	124	304	490	378	241	321	326	270	214	231	249	233	153	233
DLPFC	169	275	131	194	553	375	291	272	275	344	350	269	309	300	291	281

within-participants factor comparing the correlation coefficient ($r > 0.5$ and $r < -0.5$) revealed there was significant interaction between the addiction group, the stimulus type, and the coefficient [$F(1,18) = 10.600$, $P = 0.004$; no main effect on the addiction group, $F(1,18) = 0.395$, ns; no main effect on the stimulus, $F(1,18) = 0.543$, ns and no main effect on the coefficient, $F(1,18) = 0.182$, ns] in the right ACC. In addition, the results also showed a significant interaction between the stimulus type and the correlation coefficient [$F(1,18) = 8.325$, $P = 0.010$] in the right ACC. A planned contrast t-test (paired t-test) showed that compared with the UN, the volume of the positively correlated ($r > 0.5$) voxels between the right amygdala and the right ACC during the US task was significantly smaller ($t = 3.178$, $P = 0.011$) and the volume of the negatively correlated ($r < -0.5$) voxels was significantly larger ($t = 6.274$, $P < 0.001$) in the smokers (Table II and Fig. 5). In contrast, no significant difference in functional connectivity was seen in the nonsmokers in this ROI.

Discriminate condition

The repeated-measures analysis of variance, same to that of the indiscriminate condition, revealed there was a significant interaction between the addiction group, the stimulus type, and the correlation coefficient in the right Caudate [$F(1,12) = 4.830$, $P = 0.048$; no main effect on the addiction group, $F(1,12) = 0.981$, ns; no main effect on the stimulus, $F(1,12) = 0.603$, ns and no main effect on the coefficient, $F(1,12) = 0.106$, ns] and the right Pulvinar [$F(1,12) = 6.818$, $P = 0.023$; no main effect on the addiction group, $F(1,12) = 1.671$, ns; no main effect on the stimulus, $F(1,12) = 1.042$, ns and no main effect on the coefficient, $F(1,12) = 0.372$, ns]. However, there was no significant interaction between the stimulus type and the coefficient

in these two ROIs [the right caudate: $F(1,12) = 0.017$, ns; the right pulvinar: $F(1,12) = 0.273$, ns].

DISCUSSION

In the right amygdala, the response to the US was significantly lower than that to the UN in the smoker group, while no significant difference was found in the non-smokers (Fig. 4, Table I). Additionally, the sub-group result suggests that the difference in BOLD signal response between the US and the UN may be functionally relevant to the abuse of nicotine in smokers. Our results provide the evidence that the amygdala response to drug-related stimuli does not rely on the participant's discrimination of the stimuli. This result is consistent with previous findings that the amygdala (especially the right amygdala) is a key brain area for classical unconscious emotional learning [Etkin et al., 2004; Killgore and Yurgelun-Todd, 2004; LeDoux, 1996, 2000; Whalen et al., 1998, 2004].

The activation level in the amygdala is related to the drug craving in drug-abusers [Wilson et al., 2004]. The smoking-related visual cue can increase the amygdala activation [Due et al., 2002] and the cigarette craving [Drummond, 2001] at the same time. In this study, an amygdala activity decrease was found when smokers see masked smoking-related pictures. A recent study [McClernon et al., 2007] showed a selectively activation reduce response to smoking cue in the amygdala following a smoking cessation treatment. Therefore, the result in this study might display some possibilities in the strategies to addiction treatment.

Besides drug-related information, for the nicotine abstinent increases the smoker's anxiety level [reviewed by Morissette et al., 2007] and the smokers were restricted

from smoking at least for 2 h in this study, the anxiety may be play a role in producing current results. However, in the recent studies about unconscious fearful stimuli [Bryant et al., 2007; Etkin et al., 2004], increased anxiety is generally associated with increased amygdala activity. Therefore, it is unlikely that the reduction in amygdala activity in response to US stimuli was contributed by smokers' increased anxiety during the current study.

In the existing behavioral studies, differential functional characteristics of the drug-related stimulus at the discriminate and indiscriminate levels have also been reported. For example, Rosse et al., [1997] found that drug craving scores were correlated negatively with the number of unconscious fixations and were correlated positively with the number of conscious fixations during visual scanning. In another study, drug-users' heart rates were decelerated only when the drug-related pictures were presented at the indiscriminate level [Ingjaldsson et al., 2003]. When the images were perceived by the drug-users, their heart rates were either not decelerated [Ingjaldsson et al., 2003] or even increased [Sinha et al., 2000]. The present results demonstrated the same smoking-related stimulus generated either increased or decreased BOLD signals in the right amygdala in the discriminate and indiscriminate conditions, respectively, and are consistent with these prior behavioral results and provide convergent neural evidence supporting the hypothesis that the discriminate and indiscriminate drug-related stimulus processes are not the same. It is also similar to the subliminal and supraliminal fear process that are dissociated in the amygdala-MPFC/ACC system [Jiang and He, 2006; Williams et al., 2006b]. However, for bradycardia can be associated with either increased or decreased amygdala activity [Henderson et al., 2002; Wu et al., 1999], the functional relationship between the amygdala activity and the heart rate in this study is still to be elucidated in the future study.

This study indicates the functional connectivity between the right amygdala and the right ACC is more negative in the smoking-related indiscriminate task than in the neutral task. In previous studies, ACC was found to have direct neural connections to the amygdala [LeDoux, 1996, 2000] inhibition is an important function of ACC in normal population [Fan et al., 2003]. Additionally, a path analysis study [Stein et al., 2007] found the activity in the amygdala was suppressed by the ACC during an emotional face matching paradigm. Therefore, one possible explanation for our result is that the ACC inhibits the indiscriminate drug-related stimulus activity in the amygdala, i.e., there may be an inhibition process when drug-users view the indiscriminate drug-related stimulus. Additionally, Brody et al. [2007] found that the activation of ACC is associated to self-suppressing cigarette craving. Therefore, the observation in this study may provide some clues for finding treatment strategies for drug abuse. However, the functional connectivity analysis in our study is based on temporal correlation, which did not allow us to determine the

full extent of the pathway. Therefore, further examination using effective connectivity analysis will be needed.

Our functional connectivity result is seemly inconsistence with some recent findings (e.g. [Williams et al., 2006a]) about indiscriminate process. Williams found activation in the bilateral amygdala covaried positively with the rostral portion of the ACC, while we found significant negative correlation between the right amygdala and the right ACC. There are several apparent differences between Williams' and our studies in the experiment design. The most salient one is that we used smoking-related cue while William focused on the fearful stimulus. Additionally, the presentation of stimuli in this study (33 ms) is significantly longer than that in Williams' study (20 ms). According to Williams' viewpoint [Williams et al., 2004], the duration of about 30 ms may not provide an exhaustive test for amygdala responses to nonconscious perception, and detection without recognition may lead to uncertainty in participants and may be sufficient to engage cortical inhibitory influences on the amygdala. Therefore, the inhibition process during the indiscriminate condition in this study might be from some discriminate influence or uncertainty. However, the absence of significant correlation during the discriminate condition in smokers and during both the discriminate and indiscriminate condition in nonsmokers may mean that there is only a minimal impact of the stimulus detection.

The present fMRI study not only provides evidence supporting the neural processing of indiscriminate drug-related stimuli but also helps to resolve seemingly conflicting reports in the literature, (i.e., the effects of unawareness processing of drug-related visual input via different measurements remain controversial). In this study, the indiscriminate drug-related stimulus affected smokers' amygdala significantly, but no such effect was observed in the DLPFC and the IFG (Table I). As an important structure in the limbic system, the amygdala plays a critical role in autonomic functions (e.g., heart rate) [Henderson et al., 2004]. Therefore, the present result is consistent with the previous report [Ingjaldsson et al., 2003] that the processing of indiscriminate drug-related stimuli was closely related to autonomic functions. Conversely, the lack of indiscriminate modulation in DLPFC and IFG by indiscriminate drug-related information, combined with many previous neuroimaging studies [Blackwood et al., 2000; Kincade et al., 2005] that showed the frontal area's important role in attentional bias, provides a reasonable account for the failure to observe high-level cognitive effects from indiscriminate drug-related stimuli in previous behavior studies [Bradley et al., 2004; Franken et al., 2000; Mogg and Bradley, 2002].

There are several limitations of this preliminary study. Drug cues would normally induce a series of activities or processes including rapid physiological/autonomic responses [Carter and Tiffany, 1999; Chiamulera, 2005] and some sustained reactions, e.g. craving and mood/emotion [Franken et al., 1999; Sinha et al., 2000]. However, because this study used a blocked design with relatively low tem-

poral resolution, it was difficult to identify the relationships between each process and the dynamic activities in the amygdala. In the future, a mixed blocked/event-related fMRI design [Scheibe et al., 2006] or combined fMRI and EEG/MEG design [Babiloni et al., 2004] with some physiological and behavioral measurements [Breiter et al., 1997; McClernon et al., 2005; Wexler et al., 2001] will be used to address this open question. Another limitation is the exclusive use of the stimuli showing male smoking. Smoking has a strong social component and male smoking might be associated with a particular image. Future experiment should also include different smoking-related cues (e.g., female smoking, smoking-related materials, etc). The third one is some participants reported awareness of flickering occurring during the indiscriminate presentations. The "smidgen of consciousness" may cause a degree of subjective uncertainty about the stimulus and thereby interfere with the brain activity from indiscriminate stimuli [Williams et al., 2004]. Therefore, we cannot exclude the function of stimulus duration as one potential reason for the finding of reduced activity in the right amygdala in our study. However, we did not find same reduced activity in nonsmokers. It suggests that this subjective uncertainty effect should be minimal in this study. The fourth limitation is the difference between the indiscriminate and discriminate conditions is that the discriminate condition lacks of the mask. Many previous studies [Etkin et al., 2004; Ingjaldsson et al., 2003; Kubota et al., 2000] about unconscious processes employed unmasked stimuli as the conscious condition. Although no participants recognized any meaningful scene in the mask during the study, the potential bias due to an imbalance between the indiscriminate and discriminate stimuli should be paid attention in future. The fifth limitation is all indiscriminate scanning runs were conducted before the discriminate scanning runs in this study. Although it can prevent perceiving indiscriminate stimulus by participants, a potential order effect may be produced. The last limitation is the relatively small sample size [i.e., 10 participants (only three smokers smoked 21–30 cigarette per day) per group and only seven participants were collected discriminate data]. Therefore, the present findings need to be confirmed by large sample study in the future. However, despite the limitation that the data were collected from a small group, the difference between smoking-related cue and the neutral cue in the key brain area (e.g., the amygdala) reached the threshold (i.e., $P < 0.05$).

CONCLUSIONS

As limited number of participants, interpretations and conclusions of the this study should be treat as preliminary. We found the activity in the amygdala was modulated by the indiscriminate smoking-related stimulus in smokers. Specifically, the same smoking-related stimulus generated either increased or decreased BOLD signal in the smoker's right amygdala in the discriminate and indiscriminate conditions, respectively. This observation suggests that the amygdala plays an important role in the indiscriminate drug-related process and different neural circuits may be involved in processing discriminate and indiscriminate drug-related stimuli. Furthermore, functional connectivity results indicate the correlation between the right amygdala and the right ACC is more negative in the smoking-related indiscriminate task than in the neutral task and give a potential possibility to the existence of inhibition processes in the unawareness drug-related pathway.

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