Acetylcholine, Drug Reward and Substance Use Disorder Treatment: Intra- and Interindividual Striatal and Accumbal Neuron Ensemble Heterogeneity May Explain Apparent Discrepant Findings

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In a recent article [1], Grasing et al. extended previous work [2] on tacrine, providing new evidence that intravenous (i.v.) cocaine self-administration can also be inhibited by two other cholinesterase inhibitors (ChEIs), rivastigmine and donepezil. In these studies, tacrine administered as either bolus injections [2] or a series of chronic infusions delivered over 21 h per day when the rats were outside of cocaine self-administration sessions [3] caused dose-related reductions in cocaine-reinforced behavior. The administration of tacrine, donepezil, or rivastigmine by chronic infusion attenuated self-administration of cocaine within 20 min [1]. Interestingly, cocaine-reinforced behavior was also attenuated 1–3 days after treatment with either tacrine or donepezil but not by rivastigmine delivered as a chronic infusion. This new work suggests that cocaine-dependent individuals may benefit from having their cholinergic system stimulated by ChEIs. It is of note that tacrine is not only a ChEI but also inhibits M1 muscarinic receptors at an only 7-fold higher concentration, with respective IC\textsubscript{50} values of 223 versus 1,500 nmol/l [4] and inhibits monoamine oxidase A, albeit with a \textit{K}\textsubscript{i} of 12,500 [5], suggesting that the observed behavioral effects may not necessarily be due only to tacrine’s ChEI effects but to at least an additive effect of ChEI and...
M1 antagonism or may be more likely due to M1 blockade only; not even an Monoamine oxidase inhibitory effect contributing to the observed behavioral effect of tacrine can therefore be excluded.

We would like to emphasize that the role of acetylcholine in drug reward and in the treatment of substance use disorders is much more complex than findings of Grasing and colleagues suggest [see 6–8, for recent reviews]. The intriguing question is: does stimulation of the cholinergic system inhibit drug reward (as findings of Grasing and others suggest) or is stimulation of the cholinergic system necessary for drug reward (as we would argue on the basis of experimental data of our group and others)? Which of the various cholinergic subsystems (α4β2 or α7 nicotinic, M1/M4 vs. M2 or M5 muscarinic) mediates which aspect of substance use disorders?

The findings by Grasing and colleagues (indicating an inhibition of drug reward by stimulation of the cholinergic system, corresponding to an enhancement of drug reward by inhibition of the cholinergic system) are corroborated by the cocaine conditioned place preference (CPP) experiments of Hikida et al. [9, 10] who demonstrated a leftward shift of the cocaine dose-response curve after genetic ablation of nucleus accumbens (Acb) cholinergic interneurons [9, but see 11] and an inhibition of cocaine CPP by donepezil [10]. The findings of Grasing and colleagues are also corroborated by the inhibitory effect of systemic nicotine (s.c.) or systemic donepezil (i.p.) on cue- or methamphetamine-induced reinstatement of responding for methamphetamine, an inhibitory effect that could be counteracted by systemic administration of the nicotinic blocker mecamylamine but not of the muscarinic blocker scopolamine [12]. Finally, the work of Grasing and colleagues is corroborated by Thomsen et al. [13] who found that systemic M1- and/or M4-preferring muscarinic agonists flattened the cocaine i.v. FR1 self-administration curve.

We would argue that the findings of Grasing and colleagues are contradicted by the work of Smith et al. [see fig. 1 of 14] who ablated accumbal cholinergic interneurons with 192 IgG-saporin and demonstrated, in a fixed ratio 2 schedule of i.v. cocaine self-administration, a flattening of the descending part of the typically lambda-shaped cocaine dose-response curve. For us, this dose-effect curve flattening is most consistent with a pronounced inhibition of both the reinforcing and the rate-decreasing effects of cocaine [15]. Now one could argue that what figure 1 of Smith et al. [15] shows is a parallel leftward shift of the cocaine dose-response curve indicating an increase in the reinforcing effect of cocaine. We would opine that the variance of the data shown in figure 1 of Smith et al. is too large to settle the argument at the statistical level (e.g. by an F test to compare the goodness-of-fit of modeled dose-response relationships). The findings of the pioneering study of Wilson and Schuster [16] in rhesus monkeys is, due to the scarcity of data, even more open to debate. The authors showed that atropine increased responding for 0.2 mg/kg i.v. cocaine (which the authors stated to be on the descending part of the cocaine dose-response curve [16, p. 646]. This single-dose finding is best explained by a rightward shift of the lambda-shaped cocaine dose-response curve indicating a decrease in the reinforcing and rate-decreasing effect of cocaine [15, 17]. Finally, we would opine that the findings of Grasing et al. are also contradicted by recent findings of Deisseroth and coworkers [11, 18] who showed that optogenetic inhibition of accumbal cholinergic interneurons inhibited the acquisition/expression of cocaine CPP in mice, greatly extending previous work by a number of independent groups [19–24]. Of note, optogenetic inhibition of accumbal cholinergic interneurons did not impair fear conditioning by contextual or auditory cues [see the supporting online material of 11], strongly indicating that the effect on cocaine CPP was not due to a ‘general’ inhibition of learning and memory that has been posited as the basis of the inhibitory effect of the muscarinic receptor antagonist scopolamine on the acquisition of cocaine CPP [25]. Similarly, in the experiments by Crespo et al. [23], acquisition of an approach to food was not affected by unilateral intra-accumbens core blockade of nicotinic or muscarinic acetylcholine receptors, whereas an approach to drugs of abuse (cocaine, remifentanil, or morphine) was inhibited by local intra-accumbens muscarinic or nicotinic blockade [22], also arguing against a ‘general memory-impairing’ effect as the basis for the observed effects on drug reward. Interestingly, Thomsen et al. [13] found that the more M1- and/or M4-preferring the antagonist was, the more selective its effect on the expression of responding for cocaine versus food under an FR1 schedule of reinforcement turned out to be.

When comparing the apparently discrepant animal data, the following two experimental parameters were comparable across laboratories and, consequently, cannot explain the disparate findings: (1) a long history of self-administration training [14 vs. 1, 2, 12, 13] or (2) the rodent species [for the mouse, see 11 vs. 9, 10, 13; for the rat, see 14, 19, 22, 23 vs. 1, 2, 12].

At the human laboratory level, De La Garza et al. [26, 27] found that the choice of i.v. methamphetamine was...
not affected by rivastigmine and only 2 of 10 self-reported subjective effects [i.e., ‘anxiousness’ and ‘desire (for amphetamine)] were significantly decreased by rivastigmine [27]. A more recent report using higher doses [28] also indicates that rivastigmine reduces methamphetamine-induced subjective effects, but not self-administration in the human behavioral laboratory. These somewhat disappointing results mirror those of Grasing et al.’s [29] own human laboratory data obtained with donepezil: the positive subjective effects of i.v. cocaine were not attenuated in humans who even showed donepezil-induced increases in ‘any’ or ‘good’ drug effects induced by low – but not high – cocaine doses.

To our knowledge, there have been only two randomized controlled trials on the effect of ChEIs on drug-dependent patients. (1) Mann et al. [30] found that galantamine (a compound which was not among the ChEIs tested in the preclinical studies mentioned above) did not affect the time to relapse in abstinent alcohol-dependent patients, but was able to decrease the amount of alcohol consumed per drinking day as compared to placebo. The same group found in the same study population that,
irrespective of the participant’s intention to quit smoking, galantamine significantly decreased the cumulative amount of cigarettes smoked by 20% and decreased the number of smoking days by 15% [31]. (2) As mentioned above, the ChEI donepezil was found to prevent CPP for cocaine in mice but was ineffective for mice in which cholinergic neurons were ablated from the Acb [10]. These promising preclinical data were not corroborated in a clinical trial of cocaine-dependent subjects, in which craving and self-reported cocaine use were statistically similar in participants treated with donepezil and placebo [32]. In addition, urine levels of benzoylecgonine (the primary metabolite of cocaine) increased in the donepezil group, while they remained unchanged in the placebo condition and decreased in the other active conditions evaluated (although none were significant as compared to baseline). The data from that trial have been taken to indicate that donepezil is ineffective as a treatment for cocaine dependence.

How can we reconcile these apparently contradictory data? Based on electrophysiological findings that drug rewards versus ‘natural’ rewards engage distinct accumbal neuronal ensembles that show different firing patterns before, during, and after the presentation of the reinforcer [33, 34], and the recent optogenetic demonstration that synchronous activation of cholinergic interneurons produces dopamine release in the Acb proper [35] as well as the striatum in general [36, see 37 for an excellent editorial], we propose the following tentative unifying hypothesis for the apparently discrepant data on the role of the accumbal cholinergic system. Various ensembles of accumbal cholinergic interneurons dedicated to a very specific reinforcement scenario (i.e., a ‘psychostimulant self-administration’ ensemble vs. a ‘social interaction’ ensemble vs. a ‘food’ or even a ‘highly palatable food’ ensemble that responds to reinforcers such as sweetened condensed milk) mediate motivated behavior toward the various reinforcers, with the drug reward-associated cholinergic interneuron ensembles being especially sensitive toward neuroplastic stimulation by drugs of abuse. Therefore, in well-trained cocaine self-administering animals, boosting cholinergic transmission of non-drug-reward-dedicated accumbal cholinergic interneurons may shift the balance away from drug seeking toward alternative rewards and it may very well depend on the ensembles that were actually targeted by a certain ablation technique whether inhibition or stimulation of drug reward/reinforcement can be obtained [compare 14 vs. 9, 10]. Which cholinergic interneuron ensembles were preferentially activated by the arguably very high ChEI doses employed by Grasing et al. to inhibit i.v. cocaine self-administration remains to be determined.

We tested our hypothesis, i.e., that different small neuron ensembles in the Acb mediate drug versus ‘natural’ reward, in our paradigm of CPP reversal by dyadic social interaction between gender (i.e., male- and weight-matched Sprague-Dawley rats [38–42]. Briefly, in this paradigm rats are first trained to acquire CPP for cocaine. Following cocaine CPP acquisition, the animals’ CPP for cocaine is either (1) extinguished by saline being given in the previously cocaine-paired chamber (cocaine extinction), with saline injections remaining paired to the previously saline-paired chamber or (2) rats are counterconditioned by social interaction being paired with saline injections given in the chamber previously paired with saline, while cocaine CPP extinction with saline is being performed in the chamber previously paired with cocaine (social interaction counterconditioning). After four such CE or SIC training and testing cycles, all rats are exposed to an additional cocaine conditioning trial. Twenty-four hours later, i.e., in an essentially cocaine-free state [22], the rats are tested one more time for CPP to determine if cocaine CPP has been reacquired or not by the one training session the day before (reacquisition of cocaine CPP).

A detailed experimental time line can be found in figure 1 of Fritz et al. [38]. We could previously show that SIC inhibits reacquisition/reexpression and is also able to reverse the reacquisition-associated activation (i.e., expression of the immediate early gene Zif268) in a variety of brain areas commonly associated with reward seeking, i.e., the Acb shell (AcbSh), central and basolateral amygdala, and the ventral tegmental area [38]. The pattern observed in the AcbSh was also found in the Acb core (AcbC), albeit to a nonsignificant degree [38]. Of importance for the present experiments, the AcbC portion that was investigated in our previous study [38] lay immediately lateral and ventral of the anterior commissure (AcbC lateral), whereas the investigated AcbSh portion was located medial of the anterior commissure and medial of the AcbC subregion surrounding the anterior commissure (AcbC medial). In the present study, we repeated these experiments in a different group of rats, taking care to count the Zif268-immunoreactive (Zif268-IR) nuclei in a precisely defined corridor at the exact dorsoventral level of the anterior commissure, separating the medial AcbSh (width, 300 μm) from the medial AcbC (width, 200 μm) and the lateral AcbC (width, 200 μm). The widths of the respective corridors were based on differential calbindin-IR as shown by Paxinos et al. [43]. It is of note that our medial AcbSh corridor ended about 100 μm lateral of
the medial AcbSh boundary so as to avoid counting Zif268-IR neurons in the adjacent islands of Calleja in the more rostral parts of the AcbSh or the adjacent vertical limb of the diagonal band in the more caudal portions of the AcbSh [44]. Taking into account the work by Meredith et al. [45] who had reported on an anteroposterior (AP) gradient with respect to accumbal cholinergic interneuron density, we took slices from the AP locations of +2.2, +1.7, and +1.0 mm relative to bregma [44] as precisely as our anatomical landmark-based orientation allowed.

The results were surprising (table 1; fig. 1). Not only did we find no AP gradient with respect to the density of Zif268-IR neurons, but saw that individual animals presented with three different types of Zif268 activation patterns: diffuse, patchy, or mixed. Furthermore, the location of Zif268-IR neuron ensembles (patches) varied with respect to the boundary between the medial AcbSh and the medial AcbC so that it can easily be imagined that an experimenter who is not entirely precise with respect to the mediolateral boundaries of the medial AcbSh and the medial AcbC could easily allocate the activated neuron ensemble to the medial AcbSh in one instance and to the medial AcbC in another (see lower left panel of fig 1). The corresponding densities of Zif268-IR nuclei for both treatment groups are shown for every AP position of each animal in table 1.

Overall, the present experiments (table 1) confirm our previous findings [38] that the amount of reacquisition-associated Zif268 activation after CE is higher than after SIC.

In contrast to our previous findings, however, we did find the expected CE activation reversal by SIC at the highest level of statistical significance in the lateral AcbC (which had shown only a nonsignificant activation reversal in our previous study [38]) and not in the medial AcbSh (which had reached statistical significance in our previous study [38] but not in the present experiments). The medial AcbC activation reversal did not reach statistical significance, but significance could still be seen in the AcbC when the values of the medial and lateral portions of the AcbC were combined, using all 15 individual slices for the calculation (SEM1; table 1).

At the behavioral level, CE produced longer times spent in the cocaine-associated chamber as compared to SIC (p = <0.0001, table 2), corroborating our previous findings [35].

Three obvious differences between our previous [38] and our present study were that (a) we had previously immunolabeled the Zif268-activated neurons with a biotin-avidin-diaminobenzidine color reaction whereas we used a fluorescent secondary antibody in the present experiments, that (b) different experimenters performed the treatment-blind counting, and that (c) in the present study, precisely defined counting corridors were at the same dorsomedial level as and contiguous with the anterior commissure whereas in our previous study AcbSh and AcbC regions of interest were ventral to but contiguous with the anterior commissure. With respect to argument (a), i.e., that different immunolabeling methods may have caused the different numerical and statistical results, we would counter that differences between the biotin-avidin-diaminobenzidine versus the fluorescence signal were nonsystematic, arguing against a generally higher amplification with the biotin labeling. Accordingly, the signal difference between CE and SIC treatment for the medial AcbSh had been 3.0 (i.e., 847/286 Zif268-IR nuclei per mm²) in the previous study [38] whereas it was 1.78 (i.e., 254/166 Zif268-IR nuclei per mm²). In the lateral AcbC, the signal difference had been 1.3 (i.e., 593/464) in our previous study and was 1.72 (259/151) in the present study, again a nonsystematic difference. Only the CE-associated Zif268 activation in the medial AcbSh was considerably more pronounced in the previous study, also arguing against the immunolabeling method being the source of this discrepancy. With respect to argument (b), i.e., that the different experimenters may be the major source of the discrepancy, we would argue that, whenever systematically studied within the present experimenter group, different experimenters always obtained the same activation pattern even if their absolute counts differed by a factor of up to 1.7 (Materials and Methods, Zif268 immunostaining). The fact that the regions of interest in our previous study were slightly more ventral than those of the present study would support our argument that in future studies, Acb subregions need to be much more precisely defined spatially. Sample size differences (i.e., 5 animals per treatment group investigated in the present study vs. 8 animals in our previous one [38], with 3 slices taken per animal in both studies) are, in our opinion, too small to account for the pronounced difference in AcbSh counts between our previous [38] and the present study. Statistical significance was certainly increased by our not averaging the 3 slices obtained for each animal before pooling them for statistical comparisons. However, table 1 shows that regardless of the pooling approach (i.e., the individual-slice-pooling approach, SEM1, or the preaveraging approach, SEM2; table 1), we could not confirm the big CE signal difference in the AcbSh demonstrated in our previous study [38] in our present study. Statistical significance was certainly increased by our not averaging the 3 slices obtained for each animal before pooling them for statistical comparisons.
### Table 1. Reacquisition/reexpression of cocaine CPP after extinction or SIC: Zif268 immunohistochemistry

#### a. Zif268-IR nuclei per mm² at reacquisition after CE

| Animal | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral |
|--------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|
| 1      | 335          | 157         | 279          | 151          | 442         | 382          | 401          | 258         | 235          | 6           | 72          | 94           |
| 2      | 269          | 178         | 177          | 241          | 33          | 185          | 6           | 72          | 94           | 111         | 27          | 219         |
| 3      | 161          | 300         | 243          | 179          | 154         | 215          | 111         | 27          | 219         |             |             |             |

#### b. Zif268-IR nuclei per mm² at reacquisition after SIC

| Animal | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral | AcbSh medial | AcbC medial | AcbC lateral |
|--------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|-------------|--------------|--------------|
| 4      | 680          | 310         | 359          | 362          | 167         | 484          | 249          | 96          | 308          |             |             |             |
| 5      | 297          | 256         | 131          | 294          | 344         | 273          | 78           | 389         | 304          |             |             |             |

#### c. Zif268-IR nuclei per mm² at reacquisition after CE/SIC

| Animal  | AcbSh medial | AcbC medial | AcbC lateral | Acb | Acb | Acb | Acb | Acb | Acb | Acb | Acb | Acb | Acb | Acb |
|---------|--------------|-------------|--------------|----|----|----|----|----|----|----|----|----|----|----|
| 6      | 296±75       | 286±83      | 299±44       | 584±121 | 880±59 | 265±19 | 149±33 | 196±81 | 345±62 | 610±81 |   |
| 7      | 172±83       | 94±43       | 152±29       | 247±57 | 419±132 | 31±8 | 94±46 | 106±62 | 201±89 | 232±83 |   |
| 8      | 151±20       | 161±79      | 226±92       | 387±86 | 537±100 | 234±27 | 90±29 | 94±40 | 184±37 | 418±28 |   |
| 9      | 431±129      | 191±63      | 384±52       | 574±86 | 1,005±201 | 305±31 | 326±123 | 162±60 | 488±82 | 792±58 |   |
| 10     | 223±73       | 330±39      | 236±53       | 566±92 | 789±66 | 243±35 | 292±168 | 194±124 | 486±99 | 730±80 |   |

In the upper part of the table, Zif268-IR nuclei per mm² are shown at reacquisition after cocaine CPP extinction for 5 animals (animals 1–5) or after SIC (animals 6–10). For the first 3 animals in each group, Zif268-IR nuclei were counted at three different AP positions, i.e., +2.2, +1.7, and +1.0 mm relative to bregma [43] in the following three regions of interest: the medial AcbSh, the medial AcbC, and the lateral AcbC lateral. In addition, the counts for the medial and lateral AcbC were added for each slice (AcbC). Finally, all accumbens subregions for each slice were added up to give the total Acb count. For 2 animals of each group (animals 4, 5, 9, and 10), the AP location of the slices was within the AP coordinates of +2.2 to +1.0 mm relative to bregma. Values are given as means ± standard errors of the mean (SEMs). SEMs were obtained in two different ways: as the counts for each AP position widely differed even within each animal, all 3 × 5 slices were pooled to give the mean ± SEM for a total of 15 individual slices (SEM1 based on n = 15). Additionally, we calculated the mean ± SEM by first averaging the values from the three slices obtained from each individual animal and then by calculating the overall mean for the 5 animals (SEM2 based on n = 5). p values were obtained with an unpaired one-tailed t test, the a priori hypothesis being that cocaine CPP reacquisition after SIC decreases the density of Zif268-IR neurons as compared to cocaine CPP reacquisition after CE [38].
experiments, whereas we found a slightly higher signal difference in the lateral AcbC in our present experiments than in our previous study [38].

Finally, we would like to say a word of caution. We do not think that the vast majority of the Zif268-activated neurons shown both in our previous [38] and present study represent cholinergic interneurons, as the density of the Zif268-IR nuclei is too high with respect to the density given for Acb cholinergic interneurons [45] and would correspond more closely to the abundance described for medium spiny neurons (i.e., GABAergic projection neurons) [46]. Recent optogenetic-electrophysiological approaches by two different groups [35, 36] have shown that synchronous activation of cholinergic interneurons triggers dopamine release from dopaminergic axon terminals via activation of nicotinic acetylcholine receptors. Based on these recent findings, one might argue that Acb cholinergic interneurons are not the major Acb neuron population mediating drug reward, but that these cholinergic interneurons are modulating the rewarding strength of a stimulus by enhancing dopamine release from dopaminergic terminals onto medium spiny neurons. Our experiments are only meant to demonstrate that neuron activation by drug- or natural reward-conditioned contextual stimuli may be highly patchy, i.e., restricted to small neuron ensembles which, to complicate matters, have been found in some but not all individuals. Future research clearly will have to address this issue.

To conclude, more work is necessary to arrive (1) at a satisfactory understanding of the role(s) of acetylcholine in drug use disorders and (2) at efficient patient-matched therapies based on modulating the cholinergic system. The hypotheses/explanations proposed above may be helpful in that quest.

### Materials and Methods

#### Subjects

Male Sprague-Dawley rats (150–250 g, aged 6–8 weeks) were obtained from the Research Institute of Laboratory Animal Breeding of the Medical University Vienna (Himberg, Austria) and were single housed at 24°C for 1 week before the start of the experiment. The animals received ad libitum access to tap water and pellet chow, and a 12-hour light/dark cycle with lights on from 08.00 to 20.00 h was maintained. All animals were treated according to high ethical and scientific standards of the European Union. The present experiments were approved by the Austrian National Animal Experiment Ethics Committee.

#### Place Conditioning Apparatus

Conditioning was conducted in a homemade three-chamber apparatus as described by Kummer et al. [41]. All behavioral tests were video recorded and analyzed offline with hand timers for the time spent in each compartment.

#### Conditioned Place Preference Training

All experiments were performed using halogen white light (20 W) and radio-generated white noise. During the pretraining test, rats were allowed to move freely between the three chambers for 15 min (900 s). Pretest bias for the chambers was determined as

### Table 2. Reacquisition after cocaine CPP extinction versus SIC: behavior

| Animal  | coc  | neu  | sal  | Animal  | coc  | neu  | int  |
|---------|------|------|------|---------|------|------|------|
| 1       | 362  | 275  | 263  | 6       | 283  | 226  | 391  |
| 2       | 406  | 206  | 288  | 7       | 274  | 275  | 351  |
| 3       | 426  | 238  | 236  | 8       | 266  | 158  | 476  |
| 4       | 402  | 271  | 227  | 9       | 236  | 245  | 419  |
| 5       | 378  | 236  | 286  | 10      | 319  | 247  | 334  |

Pooled data (n = 5) 397±11 244±13 260±13
p value for time in coc for CE vs. SIC <0.0001

The times (seconds) that an animal spent in each of the three compartments of the CPP box (coc = cocaine-associated chamber; neu = neutral (middle) chamber; sal = saline-associated chamber; int = social interaction-associated chamber) are given as means ± standard errors of the mean. Total test time was 900 s. The p values were obtained with an unpaired one-sided t test (see footnote to Table 1 for an explanation).
described previously [38] and cocaine was paired with the initially nonpreferred side. The pretest was followed by training for the acquisition of cocaine CPP. Animals that acquired cocaine CPP were then trained for (1) CE with saline injections in the previously cocaine-paired chamber or (2) SIC paired with saline injections in the previously saline-paired chamber. The training cycle for either CE or SIC was repeated 4 times, followed by an additional cocaine conditioning trial and a reacquisition test in a cocaine-free state. A more detailed experimental time line has previously been described by us [38].

Cocaine (hydrochloride salt, a gift from the National Institute on Drug Abuse to G.Z., corresponding to 15 mg/kg pure cocaine base in a volume of 1 ml/kg saline) or saline was injected i.p. immediately before placing the rat into the closed dedicated chamber.

Zif268 Immunohistochemistry

Two hours after the start of the cocaine reacquisition test, i.e., at the expected peak of Zif268 protein expression [47], rats were deeply anesthetized using isoflurane and intra-cardially perfused with 0.1 mol/l phosphate buffer followed by 4% paraformaldehyde dissolved in 0.1 mol/l phosphate-buffered saline (pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde overnight, then stored in 30% sucrose at 4°C until the brains sank (indicating sufficient penetration of sucrose into the brain tissue), and shock frozen in –40°C isopentane and stored at –80°C until sectioning. All serial brain sections (40 μm) were cut using a Cryostat (Leica). Sections were stored in phosphate-buffered saline containing 0.1% sodium azide at 8°C until processed for immunolabeling. Three free-floating sections (AP +2.2 mm, AP +1.7 mm, and AP +1.0 mm) from each rat (n = 5 per treatment) [according to 43] were processed simultaneously for the staining of Zif268 protein expression. Sections were washed (3 times for 5 min each) in 50 mmol/l TBS buffer containing 0.1% Triton X-100 (50 mmol/l TBS-T) and incubated for 30 min in 50 mmol/l TBS buffer containing 50 mmol/l glycine to block aldehyde groups. Then they were washed in 50 mmol/l TBS-T (3 times for 5 min each) and incubated for 1 h in 50 mmol/l TBS-T containing 2% BSA and 10% normal donkey serum (Millipore, S30-100ML). Subsequently, sections were incubated for 24 h at room temperature with the anti-Zif268 rabbit polyclonal primary antibody [1:166, Santa Cruz Biotechnology, sc-189 (C-19)] in 50 mmol/l TBS-T containing 2% BSA. Sections were washed in 50 mmol/l TBS-T for 1 h and incubated for 2 h in 50 mmol/l TBS-T containing 2% BSA and the fluorescence-conjugated secondary antibody Alexa Fluor 488 donkey anti-rabbit (1:400, Invitrogen, A21441). Afterwards, the tissue was given an additional washing in 50 mmol/l TBS for 1 h. Finally, sections were mounted onto gelatin-coated slides, air dried and coverslipped using Vectashield® Mounting Media. Brain sections were scanned using a Zeiss optical microscope equipped with a camera (Axioplan 2 Imaging) interfaced to a PC. The objective of the microscope was set to a magnification of 20x and pictures were made in the areas of interest, i.e., the medial AcbSh, the AcbC medial and the AcbC lateral. The pictures were then further processed using Fiji software by manually adjusted Yen thresholding the pictures for further analysis [48]. Finally, Zif268-IR nuclei were counted by an observer who was blind to treatment conditions using the Fiji cell counter plugin. A second experimenter, also treatment-blind, counted all slices (n = 9) from the lateral AcbC of the first 3 animals, and found the level of statistical significance to be similar (p = 0.0092) to the first experimenter (p = 0.029), although the absolute densities of Zif268-IR nuclei were different from those of the first experimenter. The second experimenter counted 384 ± 49 Zif268-IR nuclei/mm² for CE versus 200 ± 50 for SIC compared to 222 ± 26 for CE versus 332 ± 35 for SIC as counted by the first experimenter.

Data Analysis

Results are presented as group means ± standard errors of the mean. Differences in Zif268 protein expression for the two different treatment groups were analyzed using an unpaired one-tailed t test. All statistical tests were performed at a 0.05 level of significance and all statistical analyses were conducted with GraphPad Prism 4 (www.graphpad.com).

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Disclosure Statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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