RESEARCH PAPER

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Effects of scopolamine treatment and consequent convulsion development in c-fos expression in fed, fasted, and refed mice

Asli Zengin Turkmen[1]*, Asiye Nurten[1], Mine Erguven[2] and Emine Bilge[3]

[1] Department of Physiology, Faculty of Medicine, Istanbul Yeni Yuzyil University, Istanbul, Turkey,
[2] Department of Medical Biochemistry, Faculty of Medicine, Istanbul Aydın University, Istanbul, Turkey,
[3] Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey,
** Email: asli.zengin@yeniyuzyil.edu.tr*

Fasting, anticholinergics, and seizures affect c-fos activation in the brain. Additionally, antimuscarinic treated fasted animals develop convulsion soon after re-feeding. Therefore, we assessed whether c-fos expression changes in fed, fasting, and refed animals and how scopolamine treatment affects these changes. We further assessed whether there is a change in c-fos expression after convulsions. For this purpose, BALB/c mice fasted for 1, 3, 6, 12, 24 and 48 h periods were used. The animals were treated with saline or scopolamine. Half of the animals treated with saline or scopolamine were given food 20 min after injection. All animals were observed for development of convulsions for 30 min. At the end of this period, the brains of all animals were removed, and the percentage of c-fos active cells in the hypothalamus was determined immunohistochemically. Convulsions occurred within 1-48 h of fasting, after scopolamine treatment and re-feeding. Compared to fed animals, c-fos expression was not significantly changed in those undergoing different fasting periods, but significantly decreased after 12 h fasting. After animals were allowed to eat, c-fos activation significantly increased in the 1, 3, 6 and 12 refed-saline groups and decreased in the 48 refed-saline group. Scopolamine treatment in 1-24 h fasted animals increased c-fos expression, but decreased in 48 h fasted animals. Whereas convulsion development in scopolamine-treated 3, 6, 12 and 24 h refed animals suppressed c-fos expression. These results demonstrate that re-feeding and scopolamine treatment induces neuronal activity in the hypothalamus, while scopolamine induced convulsions after food intake suppressed the c-fos activity.

Key words: scopolamine, c-fos, mice, convulsion, fasting

INTRODUCTION

Immediate early genes encode transcription factors, which is initiated by apoptosis, cell growth, and mitosis (Kaina et al., 1997; Inada et al., 1998; Roche et al., 1999; Scholf et al., 2002). The protein product of the immediate-early gene c-fos has been used as a neuronal activation marker (Iwata et al., 1998). Physiological and/or pharmacological stimuli can lead to long-term adaptive responses and activate c-fos expression (Bozas et al., 1997; Chiasson et al., 1997; Minson et al., 1997; Patronas et al., 1998).

Various stressors such as light, odor, fasting, and food deprivation activate c-fos expression. Nutrition and food deprivation function as neuronal activators in different areas of the cerebrum (Angeles-Castellanos et al., 2004; 2005).

Fasted animals treated with antimuscarinic drugs, such as scopolamine and atropine, develop convulsions after re-feeding (Nurten et al., 2006, Enginar et al., 2009; 2010). Food deprivation, antimuscarinic treatment, and solid food intake are the main factors triggering these convulsions (Nurten et al., 2009). Furthermore, convulsion development occurs independently of the hypoglycemic consequence of food deprivation

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(Enginar et al., 2005). In cortical electroencephalogram recordings, epileptiform discharges were observed in antimuscarinic-induced convulsions in food-deprived mice following food intake (Nurten et al., 2006). Food deprivation for two days alters the [^{3}H]glutamate binding kinetics in the brain, which was antagonized by antimuscarinic treatment and food intake (Enginar et al., 2003). The other studies with antimuscarinic-induced convulsions showed that, many conventional and new antiepileptic drugs are ineffective in suppressing these convulsions (Enginar et al., 2005; Buget et al., 2016), whereas haloperidol, chlorpromazine, clonidine, tizanidine, and MK-801 were found to be effective (Enginar et al., 1997; 1999; 2003).

In previous studies, it has been shown that "immediate-early genes" were activated after drug-induced convulsions and kindled convulsions in the brain and spinal cord (Chiasson et al., 1997). Nevertheless, it is not clear how fasting stress, re-feeding and anticholinergic treatment, and consequent convulsions affect c-fos expression in the brain. Therefore, in the present study, we aimed to evaluate hypothalamic c-fos expression in fed, fasted, and refed mice and examine the effects of antimuscarinic treatment on c-fos expression as well as changes in c-fos expression after convulsions.

METHODS

Animals

Animals were maintained under standard housing conditions until the experiments. After weighing, male BALB/c mice (n=208) (25–30 g) were divided into three groups: fed, fasted, and refed (Fig. 1).

Half of the fed animals were injected with saline (c-sal group, n=8), and the other half was injected with scopolamine (3 mg/kg) (c-sco group, n=8). All animals were individually placed in wire mesh cages. After 20 min, they were given food pellets, about 2 g, and observed for convulsions. At 30 min after eating, the mice were decapitated.

Fasted animals (n=96) were divided into 2 groups. Half of the fasted animals (n=48) was deprived of food for 1 h (1f-sal), 3 h (3f-sal), 6 h (6f-sal), 12 h (12f-sal), 24 h (24f-sal), and 48 h (48f-sal); after fasting, the mice were injected with saline. The other half of the fasted animals (n=48) was also deprived of food for 1 h (1f-sco), 3 h (3f-sco), 6 h (6f-sco), 12 h (12f-sco), 24 h (24f-sco), and 48 h (48f-sco); after fasting, the mice were injected with scopolamine. All saline- and scopolamine-treated fasted animals were individually placed in wire mesh cages and observed for 30 min before decapitation.

Animals in the refed group (n=96) were divided into 2 groups. Half of the refed animals (n=48) deprived of food for 1 h (1r-sal), 3 h (3r-sal), 6 h (6r-sal), 12 h (12r-sal), 24 h (24r-sal), and 48 h (48r-sal) were injected with saline. The other half of the fasted animals (n=48) was also deprived of food for 1 h (1r-sco), 3 h (3r-sco), 6 h (6r-sco), 12 h (12r-sco), 24 h (24r-sco), and 48 h (48r-sco) and then injected with scopolamine. At 20 min after injection, the mice were given food pellets (2 g) and allowed to re-feed. All animals were observed for 30 min to determine the incidence and onset of convulsions. At the end of observation period, all animals were decapitated.

During the fasting periods, animals had access to water. All fasted animals were re-weighed after the fasting periods. Experiments were started at 08:00 am

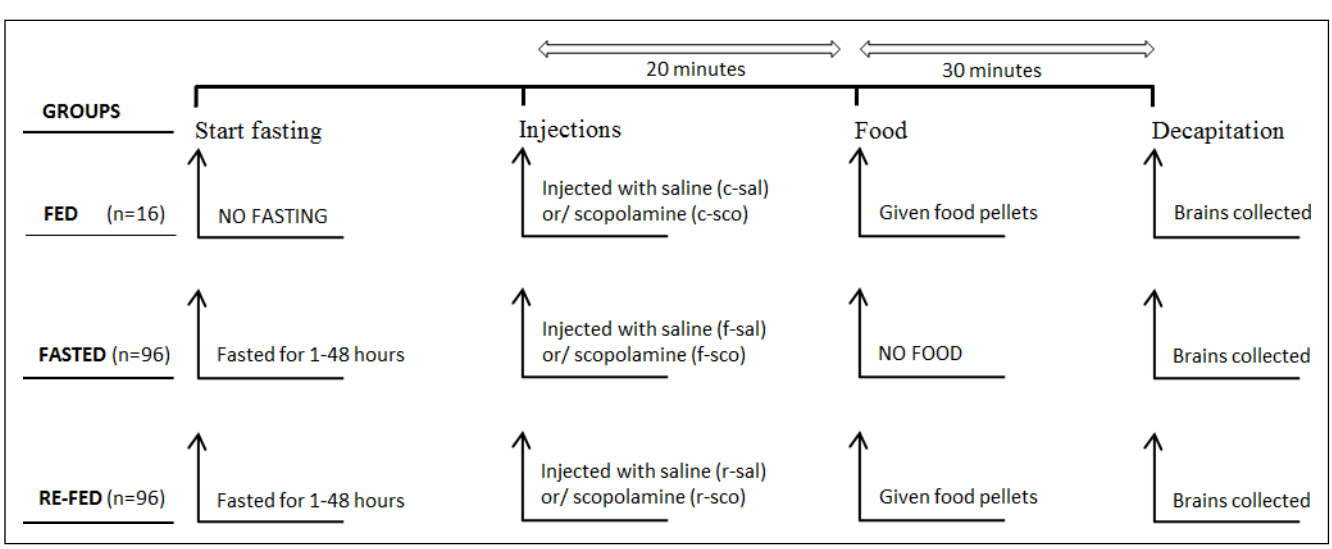

Fig. 1. The scheme of experimental design.

in a temperature-controlled (21 ± 2°C) quiet room. All observers were blinded to the treatments.

This study was approved by the Istanbul University Local Ethics Committee on Animal Experiments (NO: 32/03.05.2007). All studies were performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Mice were purchased from the Department of Laboratory Animals Science, Aziz Sancar Institute for Experimental Medicine, Istanbul University.

Convulsions

Seizure activity was scored as: (0) no difference; (1) freezing and gustatory movements; (2) forelimb clonus; (3) forelimb clonus with rearing; (4) forelimb clonus with rearing and falling; (5) generalized convulsions with rearing, falling and jumping (Enginar et al., 2003).

Stages 3, 4, and 5 were considered as convulsive responses. The time between re-feeding and stage 3 activity was defined as the onset of convulsions. The percentage of animals displaying stage 3, 4, or 5 activity was defined as the incidence of convulsions.

Tissue processing

After decapitation, all brains were removed, fixed in 10% cold formaldehyde, and embedded in paraffin blocks. Five-micrometer-thick coronal sections were prepared based on the Allen-Mouse Brain atlas. Before primary antibody incubation (1:50, anti-rabbit polyclonal c-fos antibody, Santa Cruz Biotechnology, Dallas, TX, USA), deparaffinized sections were blocked with goat serum. All sections were then incubated with biotinylated secondary antibody, a streptavidin-horse radish peroxidase complex (Santa Cruz Biotechnology, sc-2051, rabbit ImmunoCruz™ Staining System) according to the manufacturer's instructions. Color was developed with 3,3'-diaminobenzidine (DAB)/hydrogen peroxide and Gill's hematoxylin counterstaining.

Cell counts were performed in a double-blind comparison. All brain sections were investigated under a light microscope, and the numbers of c-fos positive cells were counted for each animal in two randomly chosen hypothalamic sections. All cells were counted in the total microscopic area, and the percentage of c-fos immune cells was determined.

Statistical analysis

Paired samples t-test was used to evaluate body weight loss. The onset of convulsions were evaluated by one-way analysis of variance (ANOVA) followed by Tukey tests. Fisher's exact test was used to evaluate the frequency of convulsion incidence.

Immunohistochemically labelled c-fos positive cells were evaluated by one-way ANOVA followed by Tukey test. All data expressed as the mean ± SEM.

RESULTS

Body weight loss

Mice were weighed and individually placed in wire mesh cages, and then fasted for 1 or 48 h and re-weighed at the end of the fasting period. The effect of fasting on body weight loss is shown in Table 1. At the end of the 1 or 48 h fasting periods, the mice had lost 2.5% and 18.9% of their initial body weights, respectively. Significant weight loss was detected at 6 h (t_7=6.719, P<0.001), 12 h (t_7=6.284, P<0.001), 24 h (t_7=12.142, P<0.001) and 48 h (t_7=22.581, P<0.001).

Table 1. Body weights after food deprivation.

Groups* [n]	Body weight (g) (initial)	Body weight (g) (after deprivation)	Body weight loss (%)
1 h fasting [8]	29.6 ± 0.18	28.9 ± 0.14	2.5
3 h fasting [8]	28.5 ± 0.25	27.5 ± 0.48	3.5
6 h fasting [8]	29.6 ± 0.22	28.3 ± 0.16^{a}	4.4
12 h fasting [8]	27.7 ± 0.34	25.6 ± 0.13^{a}	7.5
24 h fasting [8]	29.9 ± 0.21	25.7 ± 0.23^{a}	14.2
48 h fasting [8]	29.8 ± 0.19	24.2 ± 0.30^{a}	18.9

[n]: the number of animals. Data expressed as the mean ± SEM. * Body weight changes of the f-sal group selected to show in the table. ^{a} P<0.001, significantly different from initial body weight. Paired samples t-test.

Effects of fasting and re-feeding on c-fos expression

As shown in Fig. 2, food deprivation for 12 h decreased the number of c-fos positive cells ($F_{3,28}$=90.94, P<0.001) compared to the control (saline-treated fed animals, c-sal) group.

The c-fos expression in saline-treated refed (r-sal) animals was significantly increased after 1 h ($F_{3,28}$=97.13, P<0.001), 3 h ($F_{3,28}$=47.61, P<0.001) and 6 h fasting periods ($F_{3,28}$=37.58, P< 0.001) when compared to saline-treated fed (c-sal) animals.

Comparison of the effects of fasting and re-feeding on c-fos expression revealed that re-feeding after 1 h ($F_{3,28}$=97.13, P<0.001), 3 h ($F_{3,28}$=47.61, P<0.001), 6 h ($F_{3,28}$=37.58, P<0.001) and 12 h ($F_{3,28}$=90.94, P<0.001) significantly elevated the number of c-fos positive cells. Re-feeding after 48 h ($F_{3,28}$=7.68, P<0.05) decreased the c-fos expression, compared to 48 h fasting.

Effects of scopolamine on c-fos expression

Scopolamine injection did not significantly changed c-fos activity in fed animals.

Scopolamine administration increased the c-fos positive cell numbers in the 3f-sco ($F_{3,28}$=47.61, P<0.005) and 6f-sco animals ($F_{3,28}$=37.58, P<0.001) compared to the control (c-sco) group.

According to saline-treated fasted animals in the same periods, scopolamine, significantly enhanced the numbers of c-fos positive cells in the 1 h ($F_{3,28}$=97.13, P<0.001), 3 h ($F_{3,28}$=47.61, P<0.005), 6 h ($F_{3,28}$=37.58, P<0.001), 12 h ($F_{3,28}$=90.94, P<0.001), 24 h fasted animals ($F_{3,28}$=38.36, P<0.005) and decreased in the 48 h fasted animals ($F_{3,28}$=7.68, P<0.005) (Fig. 3).

Evaluation of convulsion development in refed mice

Scopolamine-treated 1, 3, 6, 12, 24, and 48 h fasted animals developed convulsions after re-feeding. The incidence and onset of convulsions are shown in Table 2.

Compared to the saline-treated fasted groups, incidence of convulsion significantly differed in the 12r-sco (P<0,001), 24r-sco (P<0.001), and 48r-sco groups (P<0.01). The onset of convulsions was not significantly different between the groups.

Effects of re-feeding and convulsion development on c-fos expression

c-fos expression was significantly increased in 1r-sco group ($F_{3,28}$=97.13, P<0.001), and decreased in 24r-sco group ($F_{3,28}$=38.36, P<0.005) when compared to animals in c-sco group.

Scopolamine treated fasted animals developed convulsions after re-feeding. c-fos expression in these animals were significantly decreased in the 3 ($F_{3,28}$=47.61, P<0.001), 6 ($F_{3,28}$=37.58, P<0.001), 12 ($F_{3,28}$=90.94, P<0.05) and 24r-sco groups ($F_{3,28}$=38.36, P<0.001) when compared to f-sco group animals in the same periods (Fig. 4).

DISCUSSION

The results of this study showed that fasting for 1, 3, 6, 24, and 48 h (but not 12 h) did not significantly affect c-fos expression in the arcuate nucleus. However, the number of c-fos positive cells was significantly lower in the arcuate nucleus in 12 h fasted mice. Similarly, a previous study showed that neuronal activation (c-fos

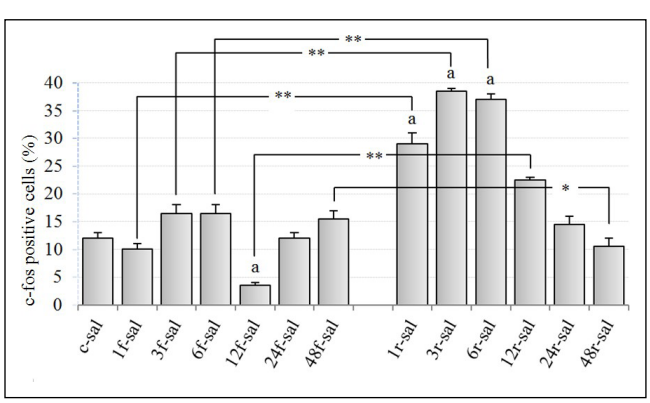

Fig. 2. Effects of fasting and re-feeding on c-fos expression. f-sal (saline-treated fasted animals); r-sal (saline-injected animals allowed to eat after food deprivation); [a] P<0.001 compared to c-sal group; * P<0.05, ** P<0.001 compared to saline-treated fasted animals.

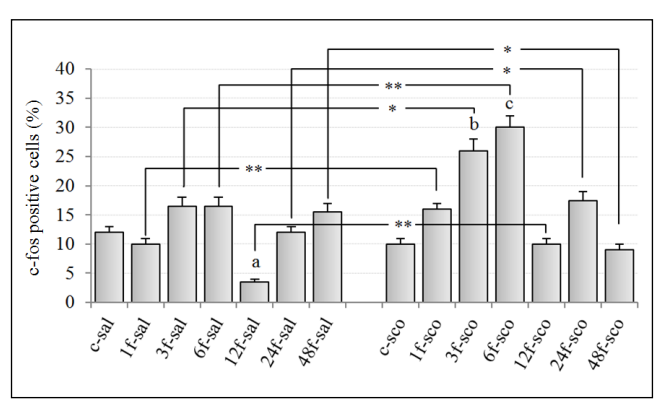

Fig. 3. Effects of scopolamine on c-fos expression. [a] P<0.05 compared to saline-treated fed animals (c-sal); [b] P<0.005, [c] P<0.001 compared to scopolamine-treated fed animals (c-sco); * P<0.005, ** P<0.001 compared to saline-injected fasted animals (f-sal).

Table 2. Incidence and onset of convulsions in scopolamine-treated fasted mice after food intake.

Groups [n]	Incidence of convulsions (%)	Onset of convulsions (min) (mean ± SEM)
*48 f-sal [8]	0	–
1 r-sco [8]	25	6.5 ± 1.5
3 r-sco [8]	50	9.0 ± 1.9
6 r-sco [8]	50	7.5 ± 2.0
12 r-sco [8]	87^{aa}	5.7 ± 0.9
24 r-sco [8]	87^{aa}	5.6 ± 2.3
48 r-sco [8]	75^{a}	5.7 ± 0.8

[n]: the number of animals. * None of the saline-treated fasted animals showed convulsions, and thus the 48f-sal group was selected to show in the table. Incidence and onset of convulsions was calculated from animals in stages 3, 4, and 5. ^{a} P<0.01, ^{aa} P<0.001, significantly different from the 48f-sal group, Fisher's exact test.

expression) did not change in rats after 3, 6, 12, and 24 h food deprivation (Timofeeva et al., 2001). Whereas, it is known that c-fos levels are increased after exposure to different stressors, including fasting. When the fasting period was 6, 12, and 24 h, it was observed that c-fos activity was increased in the brain regions related to stress, such as paraventricular hypothalamic nucleus (Timofeeva et al., 2001). A previous study showed that c-fos expression increases in animals in novel environments (Franklin and Druhan, 2000). The mice in our study were kept together for one week to acclimate to each other and the environment before fasting. Thus, the effect of the novel environment was reduced, and fasting remained as a stress factor. In addition, these differences in the results, may be arisen due to investigating only arcuate nucleus, not the stress related areas.

In the present study, experiments were started at 8:00 am, and animals were deprived of food for 1, 3, 6, 12, 24, and 48 h; therefore the 12 h fasting period was during the day.

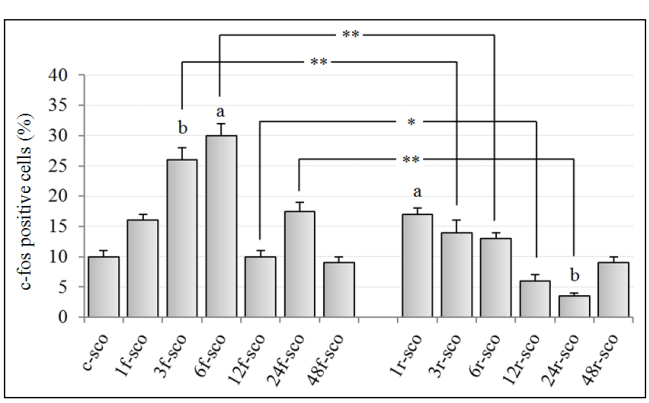

Fig. 4. Effect of re-feeding and convulsions on c-fos expression. f-sco: scopolamine-treated fasted animals; r-sco: scopolamine-treated animals, which were allowed to eat after food deprivation; ^{a} P<0.001, ^{b} P<0.005 compared to scopolamine-treated fed animals (c-sco); * P<0.05, ** P<0.001 compared to scopolamine-injected fasted animals.

Unlike our results, for food deprivation at 14 h, c-fos activity in the arcuate nucleus has been shown to increase during the night (Becksei et al., 2008). Considering that eating behavior in rats is related to the circadian rhythm (Angeles-Castellanos et al., 2004), these differences in c-fos expression may be related to the circadian rhythm.

A previous study showed that 9 h fasting increased c-fos expression in the prefrontal cortex but did not alter expression in the hippocampus (Li et al., 2015). Additionally, another study showed that fasting for 18 and 36 h increased c-fos expression in the arcuate nucleus and ventromedial hypothalamus but did not alter c-fos expression in different brain regions such as the nucleus accumbens, cingulate cortex, and insular cortex (Wu et al., 2014).

However, there was a study showed that food restriction had no effect on c-fos expression in nucleus accumbens, caudate, putamen and cingulate cortex (Carr and Kutchukhidze, 2000).

In our study, re-feeding after 1, 3, 6 and 12 h fasting significantly increased the number of c-fos positive cells in the arcuate nucleus compared to in the fed group. However, re-feeding after 24 h fasting did not alter c-fos expression compared to in the fed group. Also, c-fos expression decreased in the animals, which are refed after 48 h fasting period.

Studies investigating the effects of re-feeding on c-fos expression after 22 h of fasting showed varying results according to the hypothalamic nucleus examined. Castellanos showed that c-fos expression was increased after fasting for 3 days and after re-feeding, particularly in the paraventricular nucleus (Angeles-Castellanos et al., 2004). Additionally, Wu et al. (2014) found that c-fos expression was increased by fasting and increased further 30 min after re-feeding but decreased at 1 and 2 h after re-feeding. Our results show that neuroadaptive changes occur at 24 h after fasting and that there

is no change in c-fos expression in the arcuate nucleus at 30 min after re-feeding.

Scopolamine administration did not change c-fos expression in fed animals, but increased c-fos expression in animals fasted for 3 and 6 h compared to scopolamine-treated fed animals. This finding is fascinating because cholinergic agonists increase c-fos expression, while cholinergic antagonists suppress c-fos expression. Moreover, fasting for 12 h reverses the decrease in c-fos expression. These results indicate that the cholinergic system affects neuroadaptive modulation during fasting.

Scopolamine-treated animals developed convulsions after re-feeding and showed significantly lower c-fos expression in 3, 6, 12 and 24 re-feeding groups than the scopolamine-treated fasted group. Other studies observed changes in c-fos expression caused by the convulsant agent and in the different brain regions (Szyndler et al., 2009). In pentylenetetrazole-induced seizures, c-fos expression as shown to vary in different brain regions (Del Bel et al., 1998). Additionally, different c-fos expression patterns were observed during separate seizure circuits (Eels et al., 2004).

Convulsions developed in refed animals treated with scopolamine after 12, 24, and 48 h of fasting. Convulsions occur in 50% of refed animals after 3 h of fasting. c-fos was increased in saline-treated refed animals, while c-fos was decreased in scopolamine-treated refed animals. In fasted animals, scopolamine treatment increased c-fos levels at 3, 6 and 12 h, suggesting that c-fos was suppressed in scopolamine-treated refed animals at 3, 6 and 12 h because of the development of convulsions.

Since there are many mechanisms involved in fasting, c-fos activation in the arcuate nucleus may not be associated with food deprivation and food intake alone. Numerous studies have suggested that different stimuli in neurons are useful for increasing c-fos expression, but that increased c-fos expression does not always reflect neuronal activity (Sigan et al., 2002).

Many neurotransmitters and peptides, such as ghrelin, are known to play a role in regulating feeding behavior (Timofeeva et al., 2001). With weight loss, calorie restriction, and hypoglycemia, ghrelin secretion has been shown to increase (Cowley et al., 2003).

Additionally, during food deprivation, ghrelin secretion and neuronal activation are increased in the arcuate nucleus (Becksei et al., 2008). Another study showed that c-fos activity increased in the arcuate nucleus after systemic administration of ghrelin, a hormone that increases during hunger (Hewson and Dickson, 2000). The results of this study show that re-feeding after food deprivation induces neuronal activation in the hypothalamus.

This study evaluated hypothalamic c-fos expression in fed, fasted, and refed mice and assessed the effects of antimuscarinic treatment on c-fos expression as well as changes in c-fos expression after convulsions. Our study makes a significant contribution to the literature because we found that scopolamine-treated animals developed convulsions after re-feeding and showed significantly lower c-fos expression than the scopolamine-treated fasted group. We demonstrated that food deprivation did not affect c-fos expression except after the 12 h fasting period, whereas re-feeding significantly increased c-fos expression.

We hereby conclude that re-feeding after food deprivation induces neuronal activation in the hypothalamus.

CONCLUSION

Previous results show that mice fasting for less than 48 h develop convulsions. Food deprivation stress, but not weight loss, primarily affects convulsions. In this study, it was demonstrated that re-feeding and scopolamine treatment after fasting periods increased c-fos expression; however, convulsions suppressed c-fos expression in the hypothalamus. While evaluating the findings of the study, it should be kept in mind that the effects of scopolamine and seizures were examined separately, but the possibility of overlap on c-fos expression is difficult to distinguish.

ACKNOWLEDGEMENTS

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