Long-term decrease in immediate early gene expression after electroconvulsive seizures

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Abstract Electroconvulsive therapy (ECT) is a well-established psychiatric treatment for severe depression. Despite its clinical utility, post-ECT memory deficits are a common side effect. Neuronal plasticity and memory consolidation are intimately related to the expression of immediate early genes (IEG), such as *Egr1*, *Fos* and *Arc*. Changes in IEG activation have been postulated to underlie long-term neuronal adaptations following electroconvulsive seizures (ECS), an animal model of ECT. To test this hypothesis, we used real-time PCR to examine the effect of acute and chronic ECS (8 sessions, one every other day) on the long-term (>24 h) expression of IEG *Egr1*, *Fos* and *Arc* in the hippocampus, a brain region implicated both in the pathophysiology of depression as well as in memory function. We observed a transient increase in *Egr1* and *Fos* expression

immediately after ECS, followed by a long-term decrease of IEG levels after both acute and chronic ECS. A separate group of animals, submitted to the same chronic ECS protocol and then subjected to open field or passive avoidance tasks, confirmed robust memory deficits 2 weeks after the last chronic ECS. The possible role of IEG downregulation on long-term learning deficits observed following ECS are discussed.

Keywords *Arc* · *Egr1* · Electroconvulsive seizures · *Fos* · Memory

Introduction

Electroconvulsive therapy (ECT) is a commonly used psychiatric treatment for severe depression management, with therapeutic effects in 80 to 90 % of the patients that are resistant to treatment with chemical antidepressants (Lisanby 2007). ECT is associated with both anterograde and retrograde memory impairments (Squire 1986; Lisanby et al. 2000). In particular, electroconvulsive seizures (ECS) disrupt memory formation when delivered post-training in behavioral learning tasks (Trepel and Racine 1999). ECS induced by electrical stimuli constitute a valid animal model for ECT and have been shown to produce effects in experimental models, contributing to our understanding of the molecular mechanisms, as well as therapeutic and side effects of ECT.

Immediate early genes (IEG), rapidly induced in response to extracellular stimuli, initiate intracellular cascades that underlie long-term adaptations in neuronal gene expression (Sng et al. 2004) and synaptic metabolism (Hope et al. 1994). Such regulatory cascades may be implicated in the long-term action of antidepressant treatments, which acutely increase neurotransmitter levels and activate receptor-coupled signal transduction pathways, but require chronic administration and

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adaptations of neuronal function to achieve their therapeutic actions (Morinobu et al. 1997; Tzingounis and Nicoll 2006).

IEG early growth response 1 (*Egr1* also known as *Zif-268*, *Krox-24*, *Ngfi-a* and *Zenk*) and FBI osteosarcoma oncogene (*Fos* also known as *c-fos*) act as transcription factors (Sng et al. 2004; Knapska and Kaczmarek 2004), and the activity-regulated cytoskeleton-associated protein (*Arc* also known as *Arg 3.1*) acts as an effector IEG, thus presenting more direct effects on synaptic plasticity (Hope et al. 1994). *Egr1* upregulation is thought to initiate a program of gene regulation leading to neuronal plasticity (Chaudhuri 1997). For instance, *Egr1* has been shown to directly induce synapse-specific proteins, morphological changes after exposure to an enriched environment and other plasticity-related phenomena (Knapska and Kaczmarek 2004; Ribeiro et al. 2004; Ronnback et al. 2005). Similarly, *Fos* upregulates phospholipid synthesis and is required for neurite elongation in differentiating PC12 Cells (Gil et al. 2004). In addition, kindling-induced granule cell axon sprouting into the supragranular region of dentate gyrus is attenuated in *Fos* null mutants (Watanabe et al. 1996). Finally, *Arc* is expressed in neurons that have undergone modifications in structure and function (Hope et al. 1994; Link et al. 1995). In fact, *Arc* protein plays a key role in activity-dependent synaptic modification (Hope et al. 1994; Link et al. 1995) by way of interactions with AMPA receptors, calcium-calmodulin kinase II and actin (Hope et al. 1994; Link et al. 1995). Along the same lines, exposure to a learning paradigm or induction of long-term potentiation (LTP), typically lead to increased IEG expression immediately after the treatment; importantly, IEG expression upregulation is necessary for the maintenance and long-term memory consolidation (Clayton 2000; Waltereit et al. 2001; Knapska and Kaczmarek 2004). Thus, disturbances in IEG induction may play a role in memory dysfunction associated with ECT.

The involvement of IEG with both antidepressant properties and memory consolidation makes them an interesting class of genes with regard to possible roles in mediating ECT/ECS effects. Here we examined whether acute or chronic ECS alter the long-term (>24 h) expression of IEG *Egr1*, *Fos* and *Arc* in the hippocampus, a brain region implicated in mnemonic function as well as in the pathophysiology of depression. Besides to determine whether memory acquisition is impaired in the long-term after ECS, rats were submitted to habituation to an open-field (OF) and step-down inhibitory avoidance (IA) task after chronic ECS.

Methods

Animals

Male adult Wistar rats (60 days) were obtained from the UNESC (Universidade do Extremo Sul Catarinense,

Criciúma, SC, Brazil) breeding colony. They were housed five per cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). Molecular and behavioral experiments involved the use of 174 rats, weighing 250–300 g at the beginning of the experiment. Given the influence of behavioral tasks on IEG expression (Hope et al. 1994; Watanabe et al. 1996; Knapska and Kaczmarek 2004; Ribeiro et al. 2004), different animals were used for behavioral and gene expression experiments. Animal procedures were in strict accordance with the National Institute of Health guidelines and with the approval of the local ethics committee.

ECS treatment

Bilateral ECS was administered via pads on spring-loaded ear clip electrodes using a pulse generator (Microeletrônica Lajeadoense, Lajeado, RS, Brazil). Stimulation parameters were 150 V, 50 Hz, sine wave; with a duration of 2 s (Barichello et al. 2004). There was internal current stabilization in the pulse generator and each animal received the same current intensity. After the ECS, rats fall over to one side, curled up, and experienced tonic-clonic convulsions, were observed hind limb tonic extensions in the animals. The righting reflex was regained only after several minutes of unconsciousness. There was no mortality after ECS. Eighty-four rats were divided in to 12 equal groups ($n = 7$) and subjected to a single stimulation (acute condition) or a series of eight stimulations, applied once every 48 h (chronic condition). Hippocampal tissue was collected 0 (the animals were killed immediately after last seizure), 2, 7, 30 and 90 days after the last seizure. Sham-ECS groups were handled, respectively, as acute and chronic ECS groups and had ear clips placed, but without shock delivery.

Sample preparation and real-time PCR

For the molecular tests, animals were killed by decapitation and their brains were quickly removed. The hippocampus was manually dissected, quickly frozen on dry ice, and tissue from seven animals was pooled for each time point. One tissue pool was made for each time point. A 30 mg of frozen pooled tissue samples were homogenized with a Polytron (Fisher Scientific, Houston, TX, USA) for 30 s. Total RNA was isolated via a non-phenolic procedure (RNeasy kit, Qiagen, Hilden, NW, Germany) as described by the manufacturer. RNA quality and quantity was determined by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) followed by agarose gel electrophoresis to assess RNA integrity. A 1 µg of total RNA from each pool was reverse-transcribed into double-stranded cDNA using an oligo-dT-15 primer and

Improm-IITM Reverse Transcriptase (Promega Corporation, Madison, WI, USA) as described by the manufacturer.

Real-time PCR (qRT-PCR) experiments were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for *Arc*, *Fos* and *Egr1*, as target genes. In accordance with geNorm software (Vandesompele et al. 2002) *B2m* and *Rpl19* were chosen as the best housekeeping genes for our samples. All qRT-PCR experiments were conducted in triplicates. qRT-PCR efficiency was calculated from the slope of the calibration curve according to the equation: $E = 10^{[-1/\text{slope}]}$ as described by (Pfaffl 2004). PCR quantification was performed by converting the differences in cycle threshold values to a percentage after normalizing to the unregulated housekeeping genes as described by (Vandesompele et al. 2002). We performed a log transformation of the normalized relative gene expression levels to make the data distribution more symmetric, attributing equal weight to conditions with overexpression or underexpression (Willems et al. 2008). Finally, all samples were normalized to the chronic sham group.

Open field

Sixty animals were equally divided into 4 groups of 15 animals and subsequently chronic ECS-stimulated (or sham-ECS for control) seven (7) or fourteen (14) days before exposure to the open-field (OF) apparatus. Each animal was submitted to the OF task twice with a 24 h interval between tasks. The task was carried out in 60 × 40 cm (length and width) open-field surrounded by 50 cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided by black lines into 12 equal squares. In both sessions training (7 or 14 days after the last ECS) and testing sessions (8 or 15 days after the last ECS), animals were gently placed on the left rear square of the apparatus and allowed to explore freely for 5 min. Crossings of the black lines and rearings performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation (Reus et al. 2008).

Step-down inhibitory avoidance task

After 14 days, chronic ECS treatment (or sham-ECS for control) 30 animals ($n = 15$ each group) were exposed twice to the step-down inhibitory avoidance (IA) apparatus, with a 24-h interval between tasks. The IA apparatus consisted of a 50 × 25 × 25 cm plastic box with a front glass wall, whose floor had parallel 10 mm bronze bars. The left end of the grid was occupied by a 7 cm wide, 2.5 cm high Formica platform. The rats were gently placed

on the platform facing the rear wall, and their latency to step down onto the grid with all four paws was recorded. In the training session, after stepping down, the animals received a 0.4 mA, 2 s scrambled footshock and were withdrawn immediately from the cage. In the test session, 24 h later, the procedure was repeated, but footshock was not given. Test session step-down latency was used as a measure of retention. A ceiling of 180 s was imposed on this measure (Reus et al. 2008).

Statistical analysis

The variables were analyzed according to their distribution through Shapiro Wilk's test for normality. The homogeneity of variances among groups was assessed by the Levene test. A two-way ANOVA was used to account for treatment (acute and chronic) and temporal effects in each gene expression as well as for interaction between these factors. Tukey's test was used as a post hoc for group comparisons. In OF, groups were compared by independent samples Student's *t* test. Differences between training and testing sessions within each group were analyzed by the paired Student's *t* test. OF data were reported as mean ± SEM. In IA, groups were compared by the Mann–Whitney test. Differences between training and testing sessions were analyzed by the Wilcoxon's test. Data obtained in IA were reported as median ± interquartile ranges (25 and 75). IA data analysis data were nonparametric because this procedure involved a cutoff score. *p* values lower than 0.05 were considered to be statistically significant.

Results

Changes in IEG expression after ECS treatment

A significant decrease in gene expression was observed for all tested genes comparing chronic versus acute ECS. This decrease was 1.3-fold for *Arc* ($F = 15.895$, $p = 0.001$, $df = 1$), 1.4-fold for *Egr1* ($F = 13.113$, $p = 0.001$, $df = 1$) and 1.5-fold for *Fos* ($F = 58.169$, $p < 0.001$, $df = 1$) (Fig. 1). A similar significant time-dependent effect was found for *Arc* ($F = 18.721$, $p < 0.001$, $df = 5$), *Egr1* ($F = 28.966$, $p < 0.001$, $df = 5$) and *Fos* ($F = 63.476$, $p < 0.001$, $df = 5$) (Fig. 1). A treatment–time interaction was observed for *Arc* ($F = 6.225$, $p = 0.001$, $df = 5$) and *Egr1* ($F = 15.291$, $p < 0.001$, $df = 5$), but not for *Fos* ($F = 2.015$, $p = 0.113$, $df = 5$).

Chronic ECS significantly decreased *Arc* expression in the hippocampus 30 days after the last ECS stimuli ($p = 0.006$) in comparison to sham-treated animals (Fig. 1). The acute treatment decreased *Arc* expression 30 days ($p < 0.001$) and

90 days ($p < 0.001$) after the last ECS (Fig. 1). *Egr1* expression presented a biphasic pattern following chronic ECS. There was a twofold increase in *Egr1* expression immediately after the last chronic ECS stimulus ($p < 0.001$) (Fig. 1). Conversely, *Egr1* levels significantly decreased 7 days after treatment ($p = 0.002$), then returned to baseline levels (Fig. 1). Acute ECS promoted *Egr1* downregulation, with significant decreases observed at days 30 ($p < 0.001$) and 90 ($p < 0.001$) (Fig. 1). In both chronic and acute ECS stimulated animals *Fos* expression was significantly increased (1.7-fold, $p < 0.001$ for acute; $p = 0.001$ for chronic) immediately after treatment and decreased afterwards (Fig. 1; $p < 1$).

Fig. 1 Time-dependent immediate early genes (IEG) expression following chronic and acute (single) electroconvulsive seizures (ECS) in 84 rats ($n = 7$). **a** *Arc*, **b** *Egr1*, **c** *Fos*. The results are shown as mean $\pm$ sem. Chronic ECS is represented by filled markers (filled circles) and acute ECS by open markers (open circle). * $p < 0.05$ compared to acute sham group. † $p < 0.01$ compared to chronic sham group

Acute and chronic sham ECS groups did not differ significantly in *Fos* and *Arc* expression. However, a significant increase in *Egr1* levels was observed in acute versus chronic sham ECS ($p < 0.01$). This is in line with the increased expression of *Egr1* immediately after exposure to novel stimuli, with subsequent decline after repetitive exposures (Ronnback et al. 2005).

Egr1 expression was significantly decreased following chronic ECS in comparison to acute treatment, as previously reported by Jung et al. (1996). On the other hand, it is interesting to note that we observed an increase in *Egr1* expression immediately after chronic ECS but not after acute treatment. We believe that this apparent contradiction might derive from different *Egr1* expression dynamics after chronic and acute ECS administration. Indeed *Egr1* expression would increase faster after chronic stimulation, which would allow us to observe its increase; but after acute administration this increase would only be measured at a later time point. Further studies are necessary to address this issue.

Impaired mnemonic function after ECS treatment

Locomotor and exploratory activity were assessed by the number of crossings and rearings in the OF training session. Group comparisons using independent samples, Student's *t* test revealed that the spontaneous locomotor and exploratory activity in rats was significantly reduced at 7 days after ECS (Fig. 2) compared to the sham group (crossings: $F = 19.17$, $p < 0.001$, $t = 7.46$, $df = 25$; rearings: $F = 2.89$, $p < 0.005$, $t = 3.091$, $df = 25$). The impairment in the locomotor and exploratory activity was recovered in the rats that were assessed 14 days after ECS as shown in Fig. 2. However, when ECS administered rats were re-exposed to the open-field apparatus (Fig. 2) and they did not present a statistical difference between training and test sessions as shown by the paired *t* test (crossings: $t = 1.068$, $df = 13$, $p = 0.305$; rearings: $t = 0.370$, $df = 13$, $p = 0.717$), suggesting that the rats were not habituated to the open-field apparatus. Importantly, the sham group displayed a reduced number of crossings (sham: $t = 6.099$, $df = 14$, $p < 0.001$) and rearings (sham: $t = 7.32$, $df = 14$, $p < 0.001$) when re-exposed 24 h later to the apparatus, thus indicating habituation to the environment (Fig. 2).

In the step-down inhibitory avoidance task, the sham group (Wilcoxon test: $Z = 2.528$, $p = 0.011$) displayed an increased step-down latency when re-exposed to the apparatus after a gap of 24 h compared to the latency displayed in the training session (Fig. 3), which indicates that memory was acquired for this task. Importantly, there were no significant differences among groups in the training session of the inhibitory avoidance task (Mann–Whitney: $U = 99.5$,

Fig. 2 Habituation to open field after the last ECS in 60 rats ($n = 15$). **a** Number of crossings, **b** or rearings 7 days after the last ECS. **c** Number of crossings, **d** or rearings 14 days after the last ECS. Data are reported as mean $\pm$ SEM in training and test sessions. $*p < 0.05$ as compared to sham group

$p = 0.202$) (Fig. 3). However, ECS-administered animals significantly decreased the latency to step-down on the test session compared to sham group (Mann–Whitney: $U = 49$, $p < 0.001$).

Discussion

ECT remains one of the most effective treatments for major depression. Like chemical antidepressants, ECT exerts its clinical effects only after repeated administration, suggesting that long-term adaptations at the level of gene expression and neuronal metabolism might be involved. To our knowledge, the current study is the first to analyze IEG expression following chronic and acute ECS stimulation for a period longer than 24 h. In the present study, we

showed a twofold increase in *Egr1* expression immediately after the last chronic ECS stimulus. Conversely, *Egr1* levels significantly decreased 7 days after treatment, then returned to baseline levels. Acute ECS promoted *Egr1* downregulation, with significant decreases observed at days 30 and 90. In both chronic and acute ECS stimulated animals, *Fos* expression was significantly increased immediately after treatment and decreased afterwards. As could be seen, there was a transient increase in *Egr1* and *Fos* expression immediately after ECS.

Our data corroborate the extensive evidence of transient IEG increase following ECS (Lanahan et al. 1997; Wallace et al. 1998). Furthermore, our results revealed a significant long-term decrease in hippocampal IEG expression after ECS. A similar decrease in mRNA and protein expression has previously been reported within 24 h of ECS in the case of *Fos* (Winston et al. 1990; Tsankova et al. 2004) and *Arc* (Lanahan et al. 1997), but had never been shown for *Egr1* and such reduction in the abundance of IEG transcripts to levels below the baseline may reflect an active mechanism for transcriptional shut down (Link et al. 1995; Waltereit et al. 2001). Finally, IEG expression following chronic ECS was significantly decreased in comparison to acute treatment, as previously reported by (Jung et al. 1996) for *Egr1*. This finding is in line with the fact that chronic treatment desensitizes the ability of a subsequent ECS to induce IEG (Winston et al. 1990; Tzingounis and Nicoll 2006).

Chromatin remodeling at gene promoter regions has been increasingly recognized as a key modulator of gene expression that may partly mediate acute and chronic effects of ECS (Tsankova et al. 2004). Along the same line, it has been shown that the acetylation levels of histone H4

Fig. 3 Latency to step-down in the inhibitory avoidance task 14 days after the last ECS in 30 rats ($n = 15$). Data are reported as median $\pm$ interquartile range in training and test sessions. $*p < 0.05$ compared to sham group

at the promoter regions of the *Fos* gene increase transiently after chronic ECS, but decrease substantially 24 h after the last stimulus (Tsankova et al. 2004). Acute ECS had a similar profile, although the decrease was not statistically significant. Importantly, such changes in histone acetylation were correlated with the *Fos* expression pattern (Tsankova et al. 2004). Another putative mechanism might be the negative modulation of IEG expression by the AP-1 (activator protein-1) complex. The AP-1 complex, a heterodimer constituted by *Fos* and another IEG protein product, Jun, constitutes a well-known transcription factor capable of regulating gene transcription. This complex binds specifically to a site in promoter regions of genes, leading to the activation or repression of gene transcription (Tzingounis and Nicoll 2006). A previous study (Tzingounis and Nicoll 2006) described a persistent increase in AP-1 binding activity in the cerebral cortex and hippocampus of rats after chronic ECS. It is possible that the AP-1 complex may be repressing transcription of the *Fos* gene by modulating the serum response element in its promoter (Sng et al. 2004). Consistent with this hypothesis, AP1 consensus sequences were also found in the *Arc* and *Egr1* promoters (Herdegen and Leah 1998; Waltereit et al. 2001). Furthermore, *Egr1* expression is repressed by Fos and Fos11 proteins, which can form AP1 complexes (Herdegen and Leah 1998; Tzingounis and Nicoll 2006).

Some authors have argued that IEG are related to ECS antidepressant effects (Morinobu et al. 1997; Tzingounis and Nicoll 2006) while others disagree (Larsen et al. 2005). Based on the absence of a persistent increase in IEG expression after repetitive ECS, the most parsimonious explanation is that IEG expression constitutes an early response to ECS but is not related to its antidepressant properties.

In animals, ECS blocks and disrupts LTP in the neocortex and hippocampus (Trepel and Racine 1999). ECS also disrupts memory formation when delivered post-trial in learning tasks (Bohbot et al. 1996; Trepel and Racine 1999). However, we could not find any report of long term (> 24 h) memory impairment following ECS. Thus, we were first to observe that 14 days after ECS, treated animals decreased the latency to step down in the IA test session as compared to sham group. Similarly ECS stimulated animals did not habituate to the OF, as shown by similarity in the number of crossings and rearings between training and test sessions. Conversely, LTP maintenance in the hippocampus has been correlated with IEG induction (Lanahan et al. 1997; Clayton 2000; Guzowski et al. 2000; Knapska and Kaczmarek 2004). Both LTP and IEG induction are blocked by N-methyl-D-aspartate (NMDA) receptor antagonists (Clayton 2000). IEG induction occurs in hippocampus of mice and rats undergoing spatial learning in the Morris water maze (Wisden et al. 1990),

training in appetitive conditioning paradigm (Hernandez et al. 2006; Rapanelli et al. 2009), training on a two-way avoidance task (Nikolaev et al. 1992) and training on a brightness discrimination task in a Y-maze (Grimm and Tischmeyer 1997). For instance, it has been shown that the initial stages of learning require an increase in IEG expression in the hippocampus and other brain regions, while this decreases when the task has been learnt (Hernandez et al. 2006; Rapanelli et al. 2009). Finally, blockage of IEG induction by antisense oligonucleotides disrupts long-term memory and LTP maintenance (Clayton 2000; Guzowski et al. 2000). In agreement with antisense studies, *Arc* knockout mice have several memory deficits, as they do not form either long term spatial, fear, or taste memories (Hope et al. 1994). Similar findings were observed in knockout animals for *Egr1* (Jones et al. 2001) and *Fos* (Fleischmann et al. 2003). In light of the IEG effects in learning, we speculate that the long-term IEG downregulation after ECS could explain the memory deficits observed in our behavioral experiments. This could be caused by impairment in LTP maintenance and, in consequence, in learning deficits. However we should point out that we did not address this hypothesis directly and further experiments are necessary.

In line with the animal findings, one of the most striking and frequently cited effects of human ECT is the memory dysfunction that often accompanies treatment (Squire 1986; Rose et al. 2003). Indeed anterograde memory impairments are particularly severe during the first few hours after ECT (Squire 1986; Sackeim et al. 2007; Smith et al. 2010). Once the course of ECT is complete, the capacity for new learning begins to recover and usually completely restored 4 weeks after treatment (Sackeim et al. 2007; Nobler and Sackeim 2008; Smith et al. 2010). However there is evidence that learning impairments can persist up to 24 weeks (National Institutes of Health 1985; Weeks et al. 1980; Falconer et al. 2010). It is tempting to speculate that long-lasting memory impairments observed in ECT-treated patients might be, at least, partially caused by IEG downregulation in hippocampus. This notion is further supported by the fact that chronic ECS decreased IEG expression in comparison to the acute treatment. Indeed, human studies have correlated memory impairment with the amount of ECT sessions (Squire 1986). Besides this, functional neuroimaging studies point to the medial temporal lobe, which encompasses the hippocampus, as the main region involved in the anterograde amnesia following ECT in humans (Nobler and Sackeim 2008). On the other hand, we and others (Lanahan et al. 1997; O'Donovan et al. 1998; Wallace et al. 1998; Tsankova et al. 2004; Larsen et al. 2005) observed a transient increase in IEG expression shortly after ECS suggesting that other mechanisms might be involved in the short-term memory deficits following

ECT administration. Among the proposed mechanisms for these deficits is worth citing the excitotoxicity caused by NMDA receptor activation following seizures which could promote the increase in IEG expression. In support of this hypothesis, (McDaniel et al. 2006) found that the anesthetic ketamine, an NMDA antagonist, resulted in less disruption of short-term memory than etomidate anesthesia. In fact one could expect that both short- and long-term memory deficits after ECT are caused by a summation of factors since brain electrical activity, neurochemistry and blood flow are disturbed by the treatment (Nobler and Sackeim 2008; Sanchez Gonzalez et al. 2009).

Conclusion

Our results indicate that acute and chronic ECS lead to a long-term downregulation of the expression of the IEG *Arc*, *Egr1* and *Fos*. Furthermore, we showed that chronic ECS elicits a stronger IEG expression downregulation in comparison to acute ECS. We also observed significant memory impairment two weeks after ECS. Despite the lack of direct evidence we propose a role of IEG in the long-term memory deficits observed following ECS/ECT stimulation. We hope that upon further refinements and testing, this model can be used as a tool for understanding the effects of ECS signaling.

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