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BRIEF COMMUNICATION

# Upregulation of Hippocampal Extracellular Signal-Regulated Kinase (ERK)–2 Induces Antidepressant-Like Behavior in the Rat Forced Swim Test

Sergio D. Iñiguez
The University of Texas at El Paso

Lyonna F. Parise
Icahn School of Medicine at Mount Sinai

Mary Kay Lobo
University of Maryland School of Medicine

Francisco J. Flores-Ramirez and Israel Garcia-Carachure
The University of Texas at El Paso

Brandon L. Warren
University of Florida

Alfred J. Robison
Michigan State University

The hippocampus mediates responses to affect-related behavior in preclinical models of pharmacological antidepressant efficacy, such as the forced swim test. However, the molecular mechanisms that regulate escape-directed behavior in this preclinical model of despair are not well understood. Here, using viral-mediated gene transfer, we assessed how overexpression of extracellular signal-regulated protein kinase (ERK)–2 within the dorsal hippocampus influenced behavioral reactivity to inescapable swimming stress in adult male Sprague–Dawley rats. When compared to controls, rats overexpressing hippocampal ERK-2 displayed increases in the time to initially adopt a posture of immobility, along with decreases in total time spent immobile, without influencing general locomotor activity. Collectively, the results indicate that hippocampal upregulation of ERK-2 increases escape-directed behavior in the rat forced swim test, thus providing insight into the neurobiological mechanisms that mediate antidepressant efficacy.

*Keywords:* antidepressant, construct validity, ERK, FST, hippocampus, MAPK, despair, stress



Mitogen activated protein kinases (MAPKs) are postreceptor signaling proteins that are members of a superfamily of serine/threonine kinases. These proteins have maintained their signaling transduction properties across evolution and are ubiquitously distributed throughout the central nervous system (Caffrey, O'Neill, & Shields, 1999; Pearson et al., 2001). The signaling properties of the classical MAPKs, extracellular signal-regulated kinase (ERK)–1 and ERK-2 in particular, have been thoroughly characterized (Pearson et al., 2001). ERK-1 and ERK-2 are activated by both Ras-independent and Ras-dependent signaling molecules. In the Ras-dependent cascade, this small G-protein activates the enzyme Raf, which in turn phosphorylates MAPK and ERK kinase (MEK). The phosphorylated form of MEK then phosphorylates ERK-1 and ERK-2. Once activated, ERKs phosphorylate other downstream kinases and/or directly translocate into the nucleus, where they influence the activation of several transcription factors, including cyclic adenosine monophosphate–response element binding protein (CREB). ERK-mediated activation of CREB, in turn, modulates the transcription of several genes that are implicated in synaptic plasticity and mood regulation (Pittenger & Duman, 2008).

Exposure to stress, the most common risk factor preceding the development of major depressive disorder (Kendler, Karkowski, & Prescott, 1999), is associated with decreases in the expression and phosphorylation of ERK-2 within the hippocampal formation in

Sergio D. Iñiguez, Department of Psychology, The University of Texas at El Paso; Lyonna F. Parise, Fishberg Department of Neuroscience, Icahn School of Medicine at Mount Sinai; Mary Kay Lobo, Department of Anatomy and Neurobiology, University of Maryland School of Medicine; Francisco J. Flores-Ramirez and Israel Garcia-Carachure, Department of Psychology, The University of Texas at El Paso; Brandon L. Warren, Department of Pharmacodynamics, University of Florida; Alfred J. Robison, Department of Physiology, Michigan State University.

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Correspondence concerning this article should be addressed to Sergio D. Iñiguez, Department of Psychology, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968. E-mail: sdiniguez@utep.edu

postmortem human brain tissue (Dwivedi et al., 2001) as well as in animal models of chronic unpredictable stress (First et al., 2011). Furthermore, repeated administration of traditional pharmaceutical antidepressant drugs like fluoxetine (a selective serotonin reuptake inhibitor) reverses the stress-induced alterations of this signaling molecule across several brain regions (Duman, Schlesinger, Kodama, Russell, & Duman, 2007; Iñiguez, Alcantara, et al., 2014). Unfortunately, evidence of a positive relationship between hippocampal ERK1/2 activity and antidepressant efficacy is correlational and/or inconsistent (Galeotti & Ghelardini, 2012; Gourley et al., 2008). This problem stems from the fact that pharmacological tools that activate or inhibit ERK-specific isoforms, without influencing others, are not currently available (Kamakura, Moriguchi, & Nishida, 1999). Therefore, to better examine how specific upregulation of one of the ERK isoforms influences responses to inescapable stress, we selectively increased the expression of hippocampal ERK-2 using viral-mediated gene transfer (Carlezon, Nestler, & Neve, 2000; Neve, Neve, Nestler, & Carlezon, 2005) in male Sprague–Dawley rats. We then measured behavioral responses on the forced swim test (FST)—one of the most commonly used paradigms to assess antidepressant efficacy across the literature (Krishnan & Nestler, 2011; Porsolt, Le Pichon, & Jalfre, 1977). Specifically, we hypothesized that overexpression of hippocampal ERK-2 would increase escape-directed behaviors in the FST similar to the effects of traditional pharmacological antidepressants.

## Method

### Animals

A total of 21 (8-week-old) male Sprague–Dawley rats (250–275 g) were purchased from Charles River Laboratories (Hollister, CA). Rats were acclimated to the research facility for 1 week prior to experimental manipulation and were pair-housed in clear polypropylene boxes containing wood shaving bedding in an animal colony maintained at 23–25 °C (on a 12-hr light/dark cycle; lights on at 7:00 hr). Animals had access to food and water ad libitum, and experiments were conducted in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with Institutional Animal Care and Use Committee approval.

### Virus Vectors

Herpes simplex virus (HSV) vectors encoding green fluorescent protein (GFP) alone or a GFP-wildtype-ERK2 (GFP-wtERK2) were used to assess how direct upregulation of ERK-2 within the dorsal hippocampus would influence behavioral responses to inescapable swim stress. The construction of the vectors and their neurotropic effects has been thoroughly described previously (Carlezon et al., 2000; Neve, Howe, Hong, & Kalb, 1997; Robinson et al., 1996). Also, they have been validated both in vivo and in vitro (Iñiguez, Vialou, et al., 2010). The average titer of the recombinant virus stock was $4.0 \times 10^7$ infectious units/ml. Titers did not differ by $> 10\%$ among preparations. All behavioral experiments commenced 3 days after viral surgery, a time at which maximal transgene expression is caused by these HSV vectors (Barrot et al., 2002; Carlezon et al., 1998). Expression of the transgenes was limited to an area of $\sim 1$ mm$^3$ around the injection site, as we (Iñiguez, Riggs, et al., 2014; Iñiguez et al., 2008; Iñiguez, Warren, Neve, et al., 2010) and others (Carrier & Kabbaj, 2012) have previously shown.

### Animal Surgery

For stereotaxic delivery of the HSV vectors, rats were anesthetized with a ketamine/xylazine cocktail (80/10 mg/kg; intramuscular) and given atropine (0.25 mg/kg/subcutaneously) to minimize bronchial secretions; rats were then given bilateral microinjections (1.0 μl per side over 10 min of either GFP or GFP-wtERK2) into the dorsal hippocampus (coordinates from Bregma: anteroposterior: −3.8, lateral: +2, dorsoventral: −3.2 mm below dura) using a 32-gauge Hamilton syringe (Chen, Shirayama, Shin, Neve, & Duman, 2001). The local anesthetic bupivacaine was applied directly along the wound edges to minimize any potential postoperative discomfort. Surgeries were conducted between 9:00 and 13:00 hr.

### Forced Swim Test

The FST is a 2-day procedure in which rats are forced to swim under inescapable conditions. On the first day, rats are forced to swim for 15 min (Castagné, Moser, Roux, & Porsolt, 2010). Initially, they engage in escape-like behaviors but eventually adopt a posture of immobility. When retested 24 hr later, rats become immobile quickly; however, pharmacological treatment with traditional and novel antidepressant drugs, between swim exposures, significantly increases their escape-directed behaviors (Iñiguez, Warren, & Bolaños-Guzmán, 2010; Parise et al., 2013), an effect that has been correlated with antidepressant efficacy in humans (Porsolt et al., 1987). At the start of the behavioral experiment (see Figure 1a for experimental timeline), rats received bilateral intrahippocampal microinjections of either GFP or GFP-wtERK2 and were left undisturbed to recover for 2 days. Twenty-four hours later (3 days after virus infusion), rats were placed in plastic cylinders (75 × 30 cm) filled to 54 cm depth with 25 °C water and forced to swim for 15 min (Figure 1a; Day 4). At the end of this period, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min, then returned to their home cage, as previously described (Iñiguez, Warren, & Bolaños-Guzmán, 2010). All cylinders were emptied and cleaned between rats. Twenty-four hours after the initial forced swim exposure (4 days after viral infusion), rats were retested for 5 min under identical conditions, and sessions were videotaped (Figure 1a; Day 5). In this study, the latency to become immobile, total immobility, and behavioral counts (i.e., floating, climbing, and swimming) were quantified as dependent variables (Parise et al., 2013). Latency to immobility was defined as the time at which the rat first initiated a posture that did not reflect attempts to escape from the water. To qualify as immobility, this posture had to be clearly visible and maintained for $\geq 2.0$ s. Behavioral counts were taken at 5-second intervals during the 5-min retest (Detke & Lucki, 1995; Warren et al., 2011), as it is traditionally done across the literature (Castagné et al., 2010). Researchers blind to HSV treatment conditions scored behavioral assessment. Spontaneous locomotor activity was measured in a separate subgroup of animals ($n = 4$ per group) to examine whether gene transfer treatments

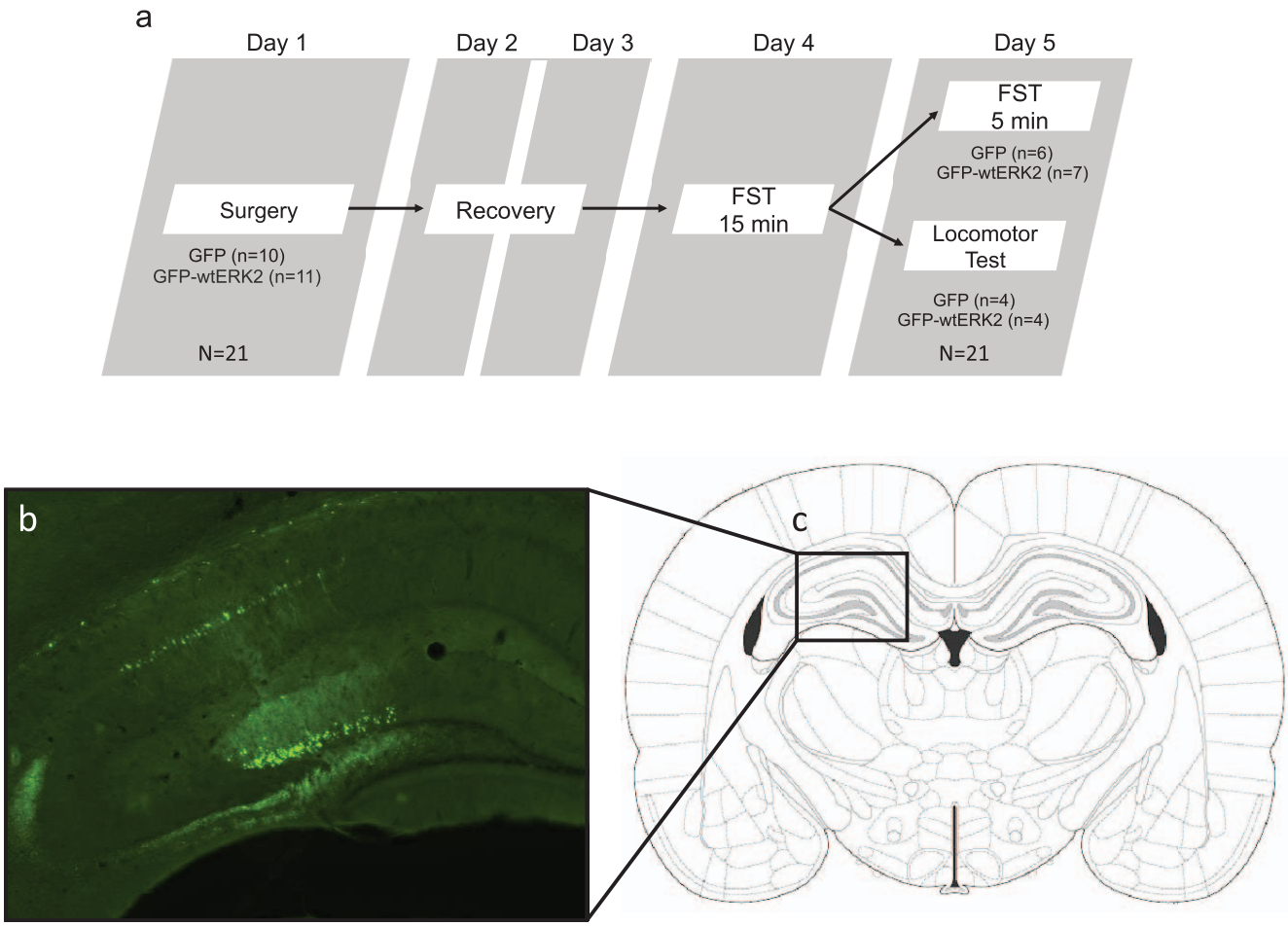


*Figure 1.* Experimental timeline and herpes simplex virus (HSV)–mediated gene transfer into the rat dorsal hippocampus. (a) Animals underwent HSV surgery (Day 1) and were given 2 days of recovery (Days 2–3). Three days postsurgery (Day 4), all rats were exposed to the first day of the forced swim test (FST, 15 min). Twenty-four hours later (Day 5), a subset of rats was reexposed to swim stress (FST, 5 min), while other rats were tested for locomotor activity. (b) Cells expressing green fluorescent protein (GFP)–wtERK2 (green, cyanine 2) fluorescence (magnification, 4×). (c) Region of the hippocampus to which microinjections of HSV vectors were targeted (anteroposterior: −3.8, lateral: +2, dorsoventral: −3.2 mm below dura). From *The Rat Brain in Stereotaxic Coordinates* (Plate 62), by G. Paxinos and C. Watson, 2007, London, UK: Academic Press. Copyright 2007 by Academic Press . Adapted with permission. See the online article for the color version of this figure.



would influence general locomotor activity during behavioral testing (Figure 1a; Day 5). Specifically, these rats were placed for 15 min in automated (75 cm diameter × 15 cm wide, four photocell beams) circular activity chambers (Med Associates, St. Albans, VT), instead of being exposed to swimming stress for 5 min (i.e., Day 2 of forced swimming).

## Histology and Transgene Detection

The injection sites were confirmed in all animals. One hour after behavioral testing, rats were given an overdose of pentobarbital and perfused transcardially with 0.9% saline, followed by cold 4% paraformaldehyde. The brains were extracted, postfixed overnight in 4% paraformaldehyde, and stored in 20% glycerol solution. Coronal sections (45 μm) through the hippocampus were taken on a microtome and stored in 0.1 M sodium phosphate buffer with 0.05% sodium azide. Sections were processed to examine the targeting of GFP expression within the hippocampus as previously described (Warren et al., 2011). Hippocampal free-floating coronal sections were processed for immunohistochemistry using a rabbit anti-GFP antibody (1:1,000; Abcam, Cambridge, MA). Adjacent sections were blocked in 3% normal donkey serum (NDS) and incubated overnight in the primary antibody mentioned above, along with 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA) and 1% NDS. Sections were incubated with antirabbit secondary antibody (1:1,000; Jackson ImmunoResearch, West Grove, PA) for 2 hr at room temperature. Stained sections were then slide mounted (Fisher Scientific), dehydrated in ethanol and Citrosolv, and coverslipped with clear DPX adhesive (Sigma, St. Louis,

MO). Slides were visualized and photographed using a fluorescence microscope and a digital camera.

## Statistical Analysis

Rats were randomly assigned to receive either GFP or GFP-wtERK2 vectors 3 days prior to behavioral testing. Data were assessed using a two-tailed Student's *t* test (Castagné et al., 2010). Data are presented as mean ± SEM. Statistical significance was defined as $p < .05$.

## Results

Figure 1b,c shows the region of the hippocampus into which microinjections of HSV vectors were targeted. Confocal microscopy (Figure 1b) revealed neurons overexpressing GFP within the dorsal hippocampus, comparable to previous work in similar (Carrier & Kabbaj, 2012) or different brain regions (Iñiguez, Alcantara, et al., 2014; Iñiguez, Warren, Neve, et al., 2010).

Figure 2 shows the effects of GFP-wtERK2 overexpression within the hippocampus on behavioral responses to forced swim stress. Specifically, GFP-wtERK2-treated rats ($n = 7$) displayed a significant increase in latency to become immobile ($t_{11} = 3.07$, $p = .005$; Figure 2a), along with a significant decrease in the time spent immobile ($t_{11} = 2.34$, $p = .01$; Figure 2b), when compared to GFP-treated controls ($n = 6$). This antidepressant-like behavioral effect was further observed when assessing behavioral climbing counts (Figure 2d), where GFP-wtERK2 rats displayed higher climbing counts when compared to controls ($t_{11} = 2.83$, $p = .008$). No differences in swimming ($t_{11} = 1.72$, $p = .056$) or floating ($t_{11} = 1.64$, $p = .06$) counts were observed between the groups as a function of HSV treatment. Importantly, differences in distance traveled were not detected as a function of hippocampal virus treatment in a separate group of rats ($n = 4$ per group) tested on a locomotor activity chamber rather than forced to swim on the second day of FST ($t_6 = 0.65$, $p = .53$; Figure 2c).

## Discussion

The goal of this study was to assess behavioral reactivity to forced swimming stress in adult male Sprague–Dawley rats after virus-mediated overexpression of ERK-2 in the dorsal hippocampus. When compared to controls, GFP-wtERK2 rats displayed increased escape directed behaviors—per increases in the latency

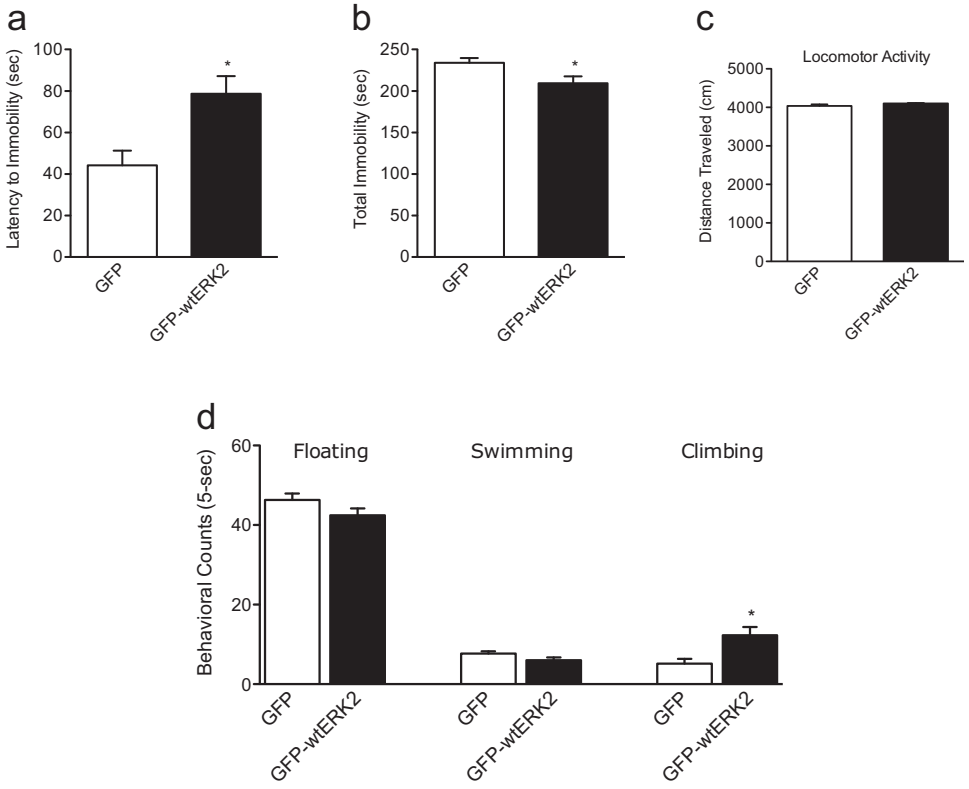


*Figure 2.* Herpes simplex virus (HSV)–mediated increases of ERK-2, in the dorsal hippocampus, alter behavioral responses in the rat forced swim test. (a) Latency to become immobile was significantly increased in rats treated with green fluorescent protein (GFP)–wtERK2 ($n = 7$) when compared with GFP controls ($^*p < .05$). (b) GFP-wtERK2-treated rats showed significantly lower total immobility than GFP ($n = 6$) treated rats ($^*p < .05$). (c) There were no group differences in locomotor activity (distance traveled, rather than swimming; $n = 4$ per group). (d) No differences in floating or swimming counts ($p > .05$) were noted between the experimental groups ($p > .05$, respectively). However, GFP-wtERK2-treated rats displayed higher climbing counts when compared to GFP controls ($^*p < .05$). Data are presented as mean (±SEM).

to become immobile, along with decreases in the total time spent immobile, and increases in the number of climbing counts. Moreover, assessment of basal locomotor activity did not reveal differences between control (GFP) and GFP-wtERK2 overexpressing rats, thus suggesting an antidepressant-like effect (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013; Castagné et al., 2010). These results support the notion that hippocampal upregulation of ERK-2 mediates antidepressant-like effects in the rat FST.

The FST has been widely utilized to screen for drugs that may induce antidepressant efficacy (Castagné et al., 2010). Accumulating evidence suggests that this model possesses high predictive (i.e., pharmacological) validity—that is, antidepressant drugs increase escape-directed behaviors by decreasing the time rodents spend immobile and/or increasing the time it takes the animal to adopt a posture of immobility (Porsolt et al., 1977). However, the molecular mechanisms that underlie such behavioral responses remain poorly understood (Krishnan & Nestler, 2008), given that traditional and novel pharmacological antidepressant agents influence a variety of neurotransmitter systems (Machado-Vieira, Henter, & Zarate, 2017).

A growing body of evidence implicates the MAPK-ERK pathway as a molecular modulator of depression-related behavior (Duric et al., 2010; Iñiguez, Vialou, et al., 2010). In general, chronic exposure to stress, in the form of forced swimming (14 days) or inescapable footshock, induces depression-related behavior and decreases ERK1/2 phosphorylation within the hippocampus (Dwivedi & Zhang, 2016; Qi, Lin, Li, Pan, & Wang, 2006). Similarly, pharmacological downregulation of ERKs, via the U0126 inhibitor, mediates a depression-related phenotype in normal rodents (Qi et al., 2009), although not in an ERK isoform-specific manner. More specifically, HSV-mediated downregulation of dorsal hippocampal ERK-2 also decreases sucrose preference (Carrier & Kabbaj, 2012)—an anhedonia-like depression-related behavioral response (Flores-Ramirez et al., 2019; Papp, Willner, & Muscat, 1991). However, inconsistent effects on hippocampal ERK phosphorylation, as a function of stress, have also been reported (Li, Linjuan-Li, & Wang, 2016; Shen, Tsimberg, Salvadore, & Meller, 2004)—likely the result of stress exposure type and/or duration.

Adding to this conceptual framework, antidepressant medications have been proposed to mediate their therapeutic effects via upregulation of ERKs within the hippocampus. For example, administration of the traditional antidepressant fluoxetine, in conjunction with daily exposure to forced swim stress, leads to increases in the phosphorylation of ERK1/2 while reversing depression-related behavior (Qi et al., 2008). Relatedly, ERK1/2 phosphorylation was upregulated in hippocampal cultures following the administration of ketamine, a fast-acting antidepressant (Lepack, Bang, Lee, Dwyer, & Duman, 2016). Here, in a similar manner to traditional and novel fast-acting antidepressant medications (Parise et al., 2013), we demonstrate for the first time that virus-mediated upregulation of hippocampal ERK-2 increases escape-directed behaviors in the rat FST (see Figure 2). Specifically, increased hippocampal activation of ERK-2 resulted in higher time (seconds) spent to adopt a posture of immobility, while at the same time reducing the total time that the rat spent immobile—behaviors that are traditionally described as antidepressant-like throughout the literature (Cryan, Valentino, & Lucki, 2005). Interestingly, HSV-mediated upregulation of ERK-2 led to increased measures of climbing counts (Figure 2d), a behavior that has been correlated with noradrenergic system activation (Detke & Lucki, 1995; Detke, Rickels, & Lucki, 1995). Thus, our results may suggest overlapping mechanisms between noradrenergic-related systems and increased hippocampal ERK-2 to mediate specific (climbing vs. swimming) escape-related behaviors. In general, these results provide construct validity to the molecular mechanisms that mediate antidepressant-like behavior in the rat FST. As such, it is possible that ERK-2 decreases despair via facilitated synaptic plasticity mechanisms (Pittenger & Duman, 2008)—since long-term potentiation leads to the predominant activation of the ERK-2 versus ERK-1 isoform (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001). Of course, given the high sequence homology between ERK-1 and ERK-2 (Boulton & Cobb, 1991), future work will be needed to evaluate whether or not ERK-1 alone mediates similar (or perhaps different) behavioral responses in this preclinical model of antidepressant efficacy since ERK-1 has been shown to influence neurobehavioral functions in other brain regions (Mazzucchelli et al., 2002).

## Conclusion

This study provides direct evidence for the role of ERK-2 within the hippocampus in mediating escape-directed behavioral responses in the rat FST. Whereas previous laboratories have provided correlative data suggesting that antidepressant medications mediate their therapeutic effects by increasing MAPK-related signaling within this brain region (Lepack et al., 2016; Qi et al., 2008), using viral-mediated gene transfer techniques, we provide a more direct approach to experimentally validate the role of ERK-2 in the induction of escape-directed behavior. Specifically, elevated ERK-2 within the dorsal hippocampus is responsible, at least in part, for mediating antidepressant-like behaviors in the adult male rat FST.

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