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Anesthesia-induced hypothermia mediates decreased *ARC* gene and protein expression through ERK/MAPK inactivation

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Several anesthetics have been reported to suppress the transcription of a number of genes, including *Arc*, also known as *Arg3.1*, an immediate early gene that plays a significant role in memory consolidation. The purpose of this study was to explore the mechanism of anesthesia-mediated depression in *Arc* gene and protein expression. Here, we demonstrate that isoflurane or propofol anesthesia decreases hippocampal Arc protein expression in rats and mice. Surprisingly, this change was secondary to anesthesia-induced hypothermia. Furthermore, we confirm *in vivo* and *in vitro* that hypothermia *per se* is directly responsible for decreased Arc protein levels. This effect was the result of the decline of *Arc* mRNA basal levels following inhibition of ERK/MAPK by hypothermia. Overall, our results suggest that anesthesia-induced hypothermia leads to ERK inhibition, which in turns decreases Arc levels. These data give new mechanistic insights on the regulation of immediate early genes by anesthesia and hypothermia.

Synaptic plasticity plays a pivotal role in memory formation and is a process dependent on rapid changes in gene expression. The mRNA of the immediate early gene *Arc* (Activity-regulated cytoskeleton-associated protein), also known as *Arg3.1*, is rapidly transported to dendritic segments, where subsequent Arc protein synthesis at synapses appears to play a significant role in the neuronal plasticity associated with learning and memory[1]. Indeed, studies have demonstrated that the disruption or suppression of *Arc* gene expression impairs the maintenance of long-term potentiation[2] as well as the consolidation of synaptic plasticity and memory[3,4].

Volatile anesthetics have been reported to suppress the transcription of a number of genes, including *Arc*[5,6]. Furthermore, amnestic doses of the commonly used intravenous anesthetic, propofol, have been demonstrated to decrease Arc protein levels[7]. However, the mechanism underlying Arc depression by anesthesia is still unknown. Thus, despite neurobehavioral evidence that anesthetics impair memory and learning[8,9], there is limited mechanistic information regarding how *Arc* gene and protein expression are altered by clinically relevant doses of anesthetics that are capable of producing surgical anesthesia.

Here, we hypothesized that exposure to clinically relevant doses of volatile or intravenous anesthetics would lead to a decrease in Arc protein levels by altering Arc translation and/or transcription. We found that Arc protein and mRNA expression were downregulated during isoflurane anesthesia. Surprisingly, this was not mediated by the exposure to isoflurane *per se*, but to the hypothermia consequent to anesthesia. We also showed that this effect was not due to a down-regulation of Arc translation by eukaryotic elongation factor 2 (eEF2), but a result of the decline of *Arc* mRNA basal levels following inhibition of Extracellular signal-Regulated Kinase/Mitogen-Activated Protein Kinase (ERK/MAPK) by hypothermia. We also confirmed that ERK inhibition is sufficient to decrease basal Arc protein levels by demonstrating that genetic ablation of MEK1 (MAPK/ERK Kinase 1, also known as MAPKK1), the kinase upstream of ERK, leads to decreases in phospho-ERK and Arc protein levels in the mouse hippocampus. Lastly, we verified our findings with propofol (an intravenous anesthetic) and exposure

to hypothermia, and confirmed low temperatures were directly responsible for the Arc protein decrease, through an effect on ERK activation and subsequent *Arc* gene transcription.

Results

Isoflurane-induced hypothermia mediates an immediate decrease in Arc protein levels in rats. Isoflurane is a halogenated ether commonly used for inhalational anesthesia in humans and animals. The rats were exposed to 1 minimum alveolar concentration (1 MAC = 1.3%) isoflurane, a clinically relevant dose that allows spontaneous ventilation[10], and does not significantly alter hemodynamic parameters in rodents with or without temperature maintainance[11–13]. The mean rectal temperatures are summarized for all groups of rats in Supplementary Table 1. Without a warming device, significant hypothermia (29.0 ± 1.5°C, n = 8) occurred at the end of isoflurane administration (IH groups). In the groups under normothermic conditions, there was no significant decrease in body temperature (IN groups).

We then analyzed hippocampal Arc levels since Arc expression in the hippocampus plays an important role the formation of long-term memory[3,4]. Under hypothermic conditions, anesthesia with 1 MAC isoflurane for 3 hours led to a decrease in Arc protein levels to ~65% of controls (Fig. 1A1; Ctl *vs.* IH). However, this decrease was transient, as Arc protein levels were not significantly different from the control group after 24 h (Fig. 1A1; Ctl *vs.* IH24h). There was no variation in β-actin levels in all the experimental conditions (Fig. 1A2 & 1B2). Interestingly, the maintenance of normothermia during isoflurane administration prevented the decrease in Arc protein levels observed under hypothermic conditions (Fig. 1B1; Ctl *vs.* IN). Our results demonstrate that Arc protein levels decrease rapidly during anesthesia; however, this reduction was surprisingly not due to isoflurane *per se*, but was mediated by isoflurane-induced hypothermia.

The reduction in Arc protein levels is not due to the modulation of Arc translation by eEF2 in rats. Arc protein levels are controlled at the translation level by eukaryotic elongation factor 2 (eEF2), an essential factor for protein synthesis. The activity of eEF2 is regulated by phosphorylation at Thr56 that inhibits its activity[14], but is associated with an increase in Arc translation[1,15,16]. Therefore, we next determined whether the decrease in Arc protein levels observed in the presence of isoflurane-induced hypothermia was due to a change in Arc translation mediated by the phosphorylation/activation of eEF2. There was a ~200% transient increase in phospho-eEF2 levels under hypothermic conditions (Fig. 2A1, Ctl *vs.* IH); however, no significant change occurred when the rats temperature was maintained at 37°C (Fig. 2B1, Ctl *vs.* IN). Moreover, phospho-eEF2 levels returned to control levels 24 hours after anesthesia, when normothermia was restored (Fig. 2A1 Ctl *vs.* IH24h). Protein levels of total eEF2 did not change during anesthesia, neither under hypothermic nor normothermic conditions (Fig. 2A2, Ctl *vs.* IH; Fig. 2B2, Ctl *vs.* IN), but increased by 25~30% 24 h later, suggesting a delayed, temperature-independent effect of isoflurane anesthesia on eEF2 levels (Fig. 2A2, Ctl *vs.* IH24h; Fig. 2B2, Ctl *vs.* IN24h). Because an elevation of eEF2 phosphorylation at Thr56 should lead to an increase in Arc translation and Arc protein levels, our results suggest that reductions in Arc levels during hypothermia are not due to a modulation of Arc translation by eEF2.

Isoflurane-induced hypothermia mediates a decrease in Arc gene expression in rats. As it was unlikely that changes in Arc translation were the cause of Arc protein reduction, we next examined *Arc* gene

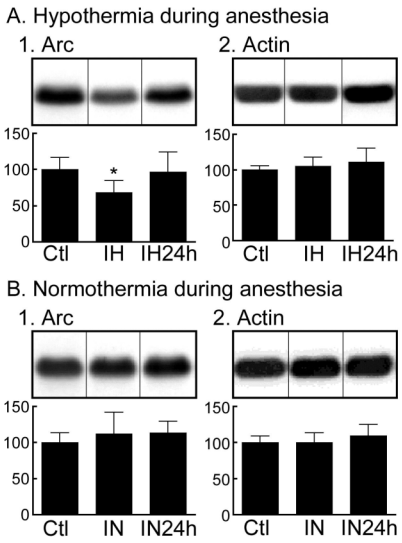

Figure 1 | Effect of isoflurane anesthesia on Arc protein levels in rats. Hippocampal Arc (1) and β-actin (2) proteins immunoblot relative band intensity (expressed as % of control) in Fischer 344 rats following exposure to isoflurane under hypothermic conditions (A) or normothermic conditions (B). Hippocampal tissues were sampled immediately at the end of anesthesia (IH: Isoflurane-Hypothermia, and IN: Isoflurane-Normothermia), or 24 h later (IH24h: Isoflurane-Hypothermia 24 h, and IN24h: Isoflurane-Normothermia 24 h). One lane from representative immunoblots for Arc and β-actin are displayed for each condition. Dividing lines represent areas where lanes from the same blot were removed and the remaining lanes were spliced together. Original unaltered data from two batches of Fisher 344 rats can be found in Fig. S1. Data are expressed as mean ± SD, ** symbol denotes a significant difference versus control with $P < 0.01$, n = 8 for all conditions.

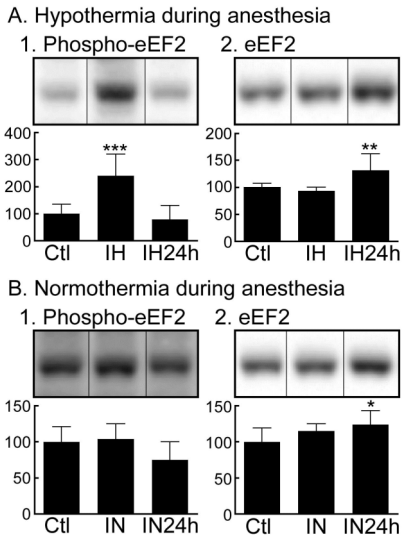

Figure 2 | Effect of isoflurane anesthesia on eEF2 and phospho-eEF2 protein levels in rats. Hippocampal phospho-eEF2 (1), and eEF2 (2) protein immunoblot relative band intensity (% of control) in Fischer 344 rats following exposure to isoflurane under hypothermic conditions (A) or normothermic conditions (B). Hippocampal tissues were sampled immediately at the end of anesthesia (IH: Isoflurane-Hypothermia, and IN: Isoflurane-Normothermia), or 24 h later (IH24h: Isoflurane-Hypothermia 24 h, and IN24h: Isoflurane-Normothermia 24 h). One lane from representative immunoblots for phospho-eEF2 and eEF2 are displayed for each condition. Dividing lines represent areas where lanes from the same blot were removed and the remaining lanes were spliced together. Original unaltered data from two batches of Fisher 344 rats can be found in Fig. S2. Data are expressed as mean ± SD, * and ** symbols denote a significant difference versus control with $P < 0.05$ and $P < 0.01$, respectively, n = 8 for all conditions.

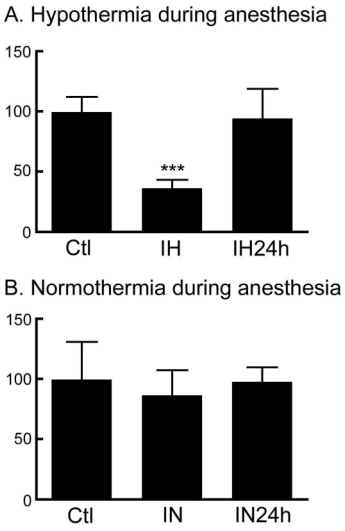

Figure 3 | Effect of isoflurane anesthesia on *Arc* mRNA levels in rats. Hippocampal *Arc* mRNA levels (normalized to β-actin mRNA and expressed as % of control) in Fischer 344 rats following exposure to isoflurane under hypothermic conditions (A) or normothermic conditions (B). Hippocampal tissues were sampled immediately at the end of anesthesia (IH: Isoflurane-Hypothermia, and IN: Isoflurane-Normothermia), or 24 h later (IH24h: Isoflurane-Hypothermia 24 h, and IN24h: Isoflurane-Normothermia 24 h). Data are expressed as mean ± SD, *** symbol denotes a significant difference versus control with $P < 0.001$, n = 8 for all conditions.

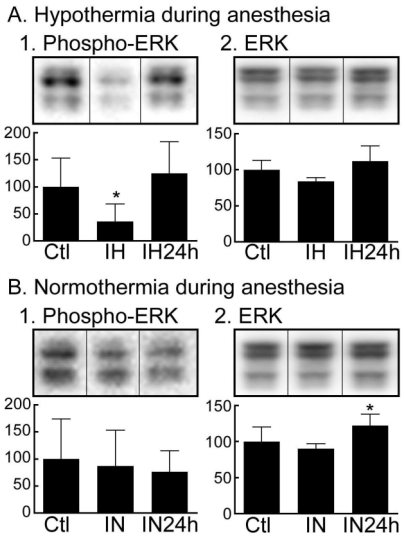

Figure 4 | Effect of isoflurane anesthesia on ERK and phospho-ERK protein levels in rats. Hippocampal phospho-ERK (1), and ERK (2) protein immunoblot relative band intensity (% of control) in Fischer 344 rats following exposure to isoflurane under hypothermic conditions (A) or normothermic conditions (B). Hippocampal tissues were sampled immediately at the end of anesthesia (IH: Isoflurane-Hypothermia, and IN: Isoflurane-Normothermia), or 24 h later (IH24h: Isoflurane-Hypothermia 24 h, and IN24h: Isoflurane-Normothermia 24 h). One lane from representative immunoblots for phospho-ERK and ERK are displayed for each condition. ERK1 (44 kDa) and ERK2 (42 kDa) were quantified together. Dividing lines represent areas where lanes from the same blot were removed and the remaining lanes were spliced together. Original unaltered data from two batches of Fisher 344 rats can be found in Fig. S4. Data are expressed as mean ± SD, * and ** symbols denote a significant difference versus control with $P < 0.05$ and $P < 0.01$, respectively, n = 8 for all conditions.

expression. Our data revealed that hippocampal *Arc* mRNA levels significantly and transiently decreased to ~35% of control immediately at the end of isoflurane exposure (Fig. 3A, Ctl *vs.* IH). In contrast, in rats that received isoflurane anesthesia under normothermic conditions, there was no significant change in hippocampal *Arc* mRNA levels (Fig. 3B, Ctl *vs.* IN). Arc mRNA levels were restored to normal levels 24 hours after anesthesia when the animals were normothermic (Fig. 3A Ctl *vs.* IN24h). Hence, hippocampal *Arc* mRNA changes, following isoflurane anesthesia in IH and IN, paralleled changes in Arc protein levels at all time points. Our results demonstrate that *Arc* mRNA levels decrease rapidly during anesthesia; however, this reduction is not due to isoflurane *per se,* but rather mediated by isoflurane-induced hypothermia.

Isoflurane-induced hypothermia leads to inhibition of ERK in rats. As we showed that *Arc* mRNA levels were diminished following anesthesia-induced hypothermia, we next investigated the mechanism underlying this decrease. It is known that synaptic activity drives *Arc* transcription in an ERK-dependent manner[17]. We thus examined the activation of ERK to see whether it could explain the decreased *Arc* transcription. ERK is 2 proteins ERK1 (44 kDa) and ERK2 (42 kDa). Here we quantified ERK1 and ERK2 together. Hippocampal phospho-ERK was reduced to ~36% of control immediately at the end of isoflurane exposure (Fig. 4A1, Ctl *vs.* IH) but returned to control values 24 h later (Ctl *vs.* IH24h). In contrast, there was no decrease in phospho-ERK in normothermic conditions (Fig. 4B1). Isoflurane treatment did not affect total ERK after 3 h (Fig. 4A2, 4B2, IH and IN), but there was a slight increase 24 h after normothermic exposure (Fig. 4B2, Ctl *vs.* IN24h). Thus, our results suggest that the isoflurane-induced hypothermia inhibits *Arc* transcription by inhibiting ERK activation.

Hypothermia induced by non-volatile anesthetics also leads to the inhibition of ERK and an Arc protein decrease in mice. As we demonstrated that isoflurane-induced hypothermia led to decreased *Arc* mRNA and protein levels, to ERK inhibition as well as to eEF2 activation, we next wanted to verify whether these results were specific to isoflurane-induced hypothermia or could be replicated with another anesthetic inducing hypothermia. We chose to test the effect of propofol, an intravenous sedative-hypnotic commonly used as an anesthetic for procedures requiring general anesthesia or conscious sedation as well as for prolonged sedation in intensive care units[18]. Moreover, we wanted to determine whether these effects were specific to male animals, so we injected propofol in female mice at 250 mg/kg, a dose that maintains spontaneous ventilation in B6 mice[19]. One hour following the injection of propofol, the rectal temperature of the mice dropped significantly from 36.5 ± 0.3°C (n = 5) to 25.4 ± 2.3°C (n = 4). Arc protein (~70% of control, Fig. 5.1) and phospho-ERK (~20% of control, Fig. 5.5) levels decreased significantly. There was no decrease in ARC level after normothermic administration of propofol (data not shown). These results are consistent with the observations made in rats following isoflurane exposure and suggest that they are mediated by anesthetic-induced hypothermia, and not consequent to hypothermia induced by a specific class of anesthetics.

Hypothermia during cold water swimming leads to the inhibition of ERK and an Arc protein decrease in mice. Although our results strongly suggest that the decreases in ERK activation and Arc protein levels are due to anesthesia-induced hypothermia, they do not irrefutably prove that they are the consequence of hypothermia *per se*. To address this issue, we induced hypothermia in mice using the cold water swimming (CWS) method[20], without any anesthetic or other pharmacological intervention. Thirty minutes after CWS, the

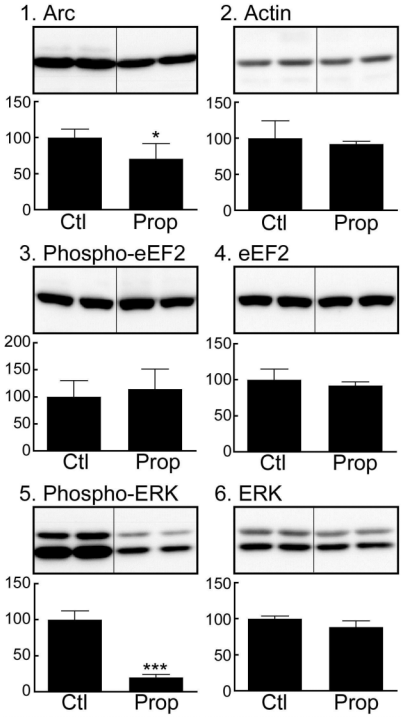

Figure 5 | Effect of propofol anesthesia on Arc, eEF2, and ERK proteins levels in mice. Hippocampal Arc (1), β-actin (2), phospho-eEF2 (3), eEF2 (4), phospho-ERK (5), and ERK (6) protein immunoblot relative band intensity (% of control) in C57BL/6J mice following exposure to propofol under hypothermic conditions. Hippocampal tissues from the propofol-anesthetized mice were obtained immediately at the end of anesthesia (Prop). ERK1 (44 kDa) and ERK2 (42 kDa) were quantified together. Two lanes from representative immunoblots for all the proteins are displayed for each condition. Dividing lines represent areas where lanes from the same blot were removed and the remaining lanes were spliced together. Original unaltered data can be found in Fig. S5. Data are expressed as mean ± SD, * and *** symbols denote a significant difference versus control with $P < 0.05$ and $P < 0.001$, respectively, n = 5 for Ctl and 4 for Prop.

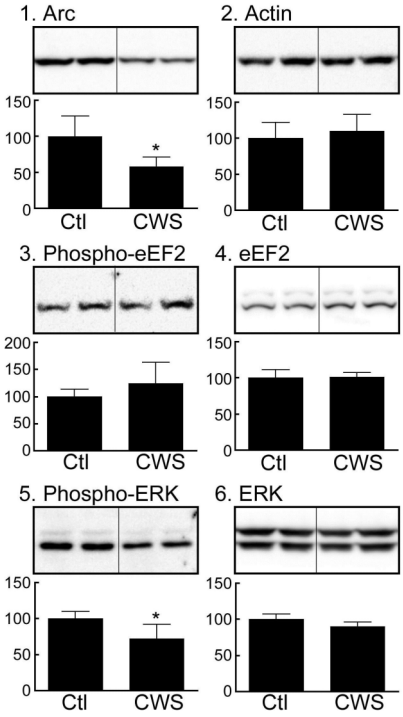

Figure 6 | Effect of cold water swimming on Arc, eEF2, and ERK proteins levels in mice. Hippocampal Arc (1), β-actin (2), phospho-eEF2 (3), eEF2 (4), phospho-ERK (5), and ERK (6) protein immunoblot relative band intensity (% of control) in C57BL/6J mice following cold water swimming (CWS). Hippocampal tissues from mice were obtained 30 min after CWS. ERK1 (44 kDa) and ERK2 (42 kDa) were quantified together. Two lanes from representative immunoblots for all the proteins are displayed for each condition. Dividing lines represent areas where lanes from the same blot were removed and the remaining lanes were spliced together. Original unaltered data can be found in Fig. S6. Data are expressed as mean ± SD, * symbol denotes a significant difference versus control with $P < 0.05$, n = 4 for all conditions.

mice rectal temperature dropped to 29.6 ± 1.7°C (n = 4); Arc protein (~53% of control, Fig. 6.1) and phospho-ERK (~72% of control, Fig. 6.5) levels decreased significantly. In summary, these results demonstrate that the activation of ERK and levels of Arc protein *in vivo* can be negatively affected by hypothermia.

Hypothermia leads to the inhibition of ERK and an Arc protein decrease in SH-SY5Y cells. We next wanted to determine whether hypothermia also mediated its effects on Arc protein levels and ERK activation *in vitro*. We chose SH-SY5Y human neuroblastoma cells for this experiment as they are known to express Arc protein[21]. Two hours following incubation at 30°C, there was a significant decrease in Arc (~58% of control, Fig. 7.1) and phospho-ERK (~29% of control, Fig. 7.5) protein levels, while eEF2 and total ERK protein levels did not change (Fig. 7.4 and 7.6, respectively, n = 6).

Suppression of ERK activity by deletion of MAPK/ERK Kinase 1 (Mek1) leads to an Arc protein decrease in mice. All our results thus far demonstrate that hypothermia leads to the inhibition of ERK and a decrease in basal Arc protein levels both *in vivo* and *in vitro*. However, these results are only correlative and do not prove that the decrease in phospho-ERK is responsible for the down-regulation of *Arc*. To provide additional evidence that ERK inactivation is directly responsible for the decreased Arc protein levels, we used mice without MEK1 and MEK2, the upstream kinases activating ERK. *Mek2−/−* mice are straight knockout mice that are viable, but do not display any effect on ERK phosphorylation, probably because of a rescue by MEK1[22]. They are used here as negative controls. Ablation of *Mek1* is lethal during embryonic development, not because of an effect in the embryos, but because of its impact on placental angiogenesis[23]. To circumvent this limitation, we used *Mek1−/−* conditional knockout mice that excise *Mek1* only in the embryos. These mice are viable and have a marked decrease in ERK phosphorylation[24]. Deletion of *Mek1* decreased phospho-ERK to ~24% of control and Arc protein levels to ~48% of control (Fig. 8.1 & 8.5, respectively), but there was no change in either actin, eEF2, phospho-eEF2 or total ERK (Fig. 8). Deletion of *Mek2* had no effect on any of the proteins studied here (Fig. 8). These results demonstrate that inhibition of ERK is sufficient to lead to decreased Arc protein levels.

Overall, our results, both *in vivo* and *in vitro*, demonstrate that the decrease in Arc and phospho-ERK protein levels during the administration of anesthesia, without control of temperature, are secondary to the direct effect of hypothermia, Furthermore, the inhibition of ERK is sufficient to induce a decrease in Arc protein levels.

Discussion

In the current study, we explored the mechanisms involved in anesthesia-mediated depression of Arc gene and protein expression. We demonstrate that it is not due to the anesthetics *per se*, but the result of hypothermia. The effect of hypothermia in animals was very robust, and reproducible i) in different species (rats or mice), ii) in

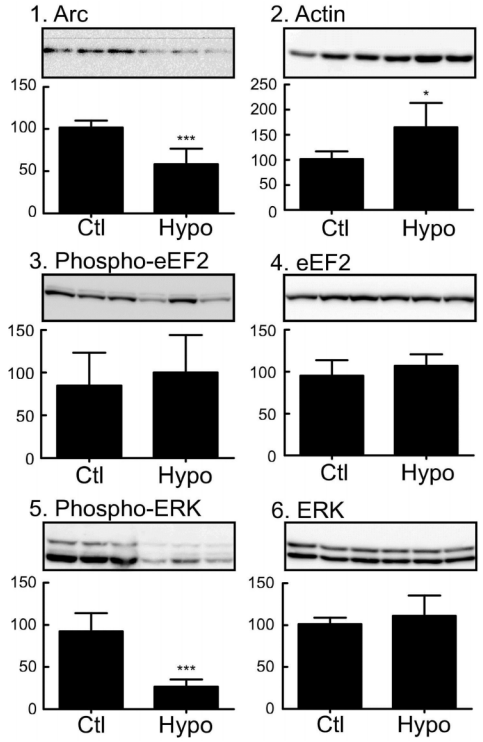

Figure 7 | Effect of hypothermia on Arc, eEF2, and ERK proteins levels in SH-SY5Y cells. Arc (1), β-actin (2), phospho-eEF2 (3), eEF2 (4), phospho-ERK (5), and ERK (6) protein immunoblot relative band intensity (% of control) in SH-SY5Y cells following exposure to hypothermic conditions. Cell lysates were sampled immediately at the end of 2 hours culture at 37°C (Ctl) or 30°C (Hypo). ERK1 (44 kDa) and ERK2 (42 kDa) were quantified together. Three lanes from representative immunoblots for all the proteins are displayed for each condition. Original unaltered data from two batches of SH-SY5Y cells can be found in Fig. S7a and S7b. Data are expressed as mean ± SD, * and *** symbols denote a significant difference versus control with $P < 0.05$ and $P < 0.001$, respectively, n = 6 for all conditions.

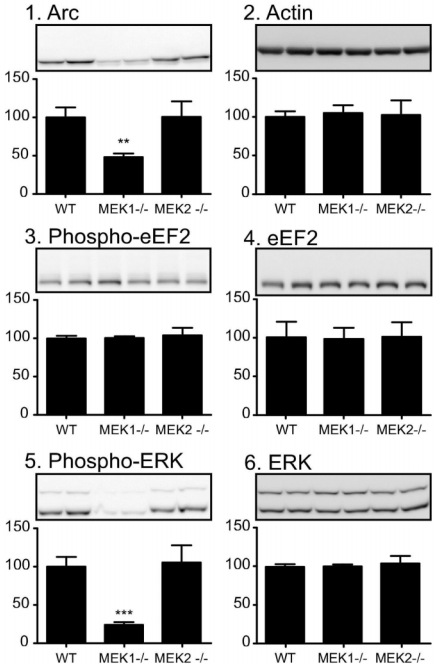

Figure 8 | Effect of *Mek*1 and *Mek*2 gene deletion on Arc, eEF2 and ERK protein levels in mice. Hippocampal Arc (1), β-actin (2), phospho-eEF2 (3), eEF2 (4), phospho-ERK (5), and ERK (6) protein immunoblot relative band intensity (% of control) in *Mek*1−/− and *Mek*2−/− mice. ERK1 (44 kDa) and ERK2 (42 kDa) were quantified together. Two lanes from representative immunoblots for all the proteins are displayed for each condition. Original unaltered data can be found in Fig. S8. Data are expressed as mean ± SD, ** and *** symbols denote a significant difference versus control with $P < 0.01$ and $P < 0.001$, respectively, n = 4 for all conditions.

males or females, iii) with different inducers of hypothermia (isoflurane, propofol, or cold-water swimming), iv) at different temperatures, v) and with different times of exposure. Importantly, Arc expression was also diminished in SH-SY5Y cells after direct exposure to hypothermia.

As anesthesia-induced hypothermia resulted in reduction of Arc protein levels, we initially investigated the molecular mechanism of this decrease by examining the phosphorylation of eEF2 protein, which regulates Arc translation. Normally, increased phospho-eEF2 should lead to enhanced Arc translation[1,15]. Surprisingly, the reduction in Arc protein correlated with an increased phosphorylation of eEF2, which suggests that eEF2-mediated control of translation is not involved in Arc protein decline during hypothermia. We also observed a robust decline in *Arc* mRNA levels, indicating that the fall in the Arc protein is due to inhibition of *Arc* transcription during anesthesia-induced hypothermia. *Arc* transcription can be regulated by ERK activity, where synaptic activity drives Arc transcription in an ERK-dependant manner[17]. Therefore, we investigated the levels of phospho-ERK, and found a significant decrease during isoflurane-induced hypothermia. More importantly, the decrease in Arc protein and phospho-ERK were reproduced following hypothermia induced by propofol or cold-water swimming in mice as well as in SH-SY5Y cells incubated at 30°C, demonstrating a direct effect of low temperatures.

Interestingly, synaptic activity induces the phosphorylation of ERK resulting in increased Arc protein levels, while inhibitors of ERK activation block stimulus-induced *Arc* gene and protein expression[1,25]. However, it was not known whether a decrease in basal ERK activation could lead to a decrease in basal Arc protein levels. Here, we show that a reduction in basal ERK activation by hypothermia correlates with depressed Arc expression, and we demonstrate that genetic reduction of ERK activation by ablation of MEK1 causes a decrease in basal Arc levels.

Overall, our results demonstrate that Arc protein and mRNA are decreased as a direct consequence of hypothermia, both *in vivo* and *in vitro*. They also suggest that hypothermia mediates its effects on Arc protein levels through inhibition of ERK.

Part of our data confirm and extend previous results showing that exposure to sevoflurane, another fluorinated hydrocarbon volatile anesthetic, leads to an immediate and persistent decrease in *Arc* mRNA in the rat brain[5]. However, in contrast to that study, the inhibitory effect of anesthesia on Arc mRNA observed in the present study was transient, returning to normal levels 24 h after isoflurane exposure. One explanation might be that these investigators did not report the temperature of the rats and used sevoflurane at 4%, which corresponds to a value as high as approximately 1.7 MAC in this species[26,27], in contrast to the 1 MAC isoflurane used in the present study. Furthermore, hypoxia, which commonly occurs at these higher anesthetic doses, has been demonstrated to significantly alter immediate-early gene expression[28]. Hence, while the distinct possibility exists that transient versus persistent changes in *Arc* mRNA could be explained by a dose dependent phenomenon, it is also possible that at higher anesthetic concentrations, they are secondary to anesthetic-induced physiologic perturbations. Indeed, Kobayashi *et al.* observed that a dose of 4.5% sevoflurane, which is slightly

higher than the final concentration used in their study (4%), resulted in death in some of their animals due to hypoxia or hypotension[5].

Hypothermia has been previously demonstrated to either increase or decrease the expression of certain immediate early genes such as *c-fos, fosB, fra-1, fra-2, c-jun* and *junB*, following an ischemic insult in the rat forebrain[29]. Interestingly, in terms of *Arc*, Otsuka *et al.* demonstrated that following transient global ischemia in normothermic mice, there was a persistent increase in hippocampal *Arc* transcription that was absent in hypothermic mice[30]. Although we did not examine *Arc* expression in the context of ischemia, our findings of a suppressant effect of hypothermia on Arc gene and protein expression are indeed consistent with those of Otsuka *et al.*, and they demonstrate that hypothermia is a potent inhibitor of Arc expression, even in the setting of conditions not specifically associated with induced Arc expression.

Hypothermia has been known for a long time to lead to memory disruption in numerous organisms including rodents[31]. In rats, memory loss has been demonstrated after cooling[31–34]. However, the mechanisms by which hypothermia produces memory disruption are not well understood. Some studies suggest that a reduction of protein synthesis resulting from a general decrease in energy-dependent cellular processes plays a role, whereas others suggest a disruption of cellular signaling events positioned upstream of transcription or translation[35]. Our results demonstrating a decrease in Arc protein and mRNA levels correlating with decreased ERK signaling following hypothermia argue for the latter hypothesis, and provide a clue to a possible mechanism underlying the disruption of memory following cooling in wide range of organisms. While it is outside of the scope of this article to test the physiological consequences of our observations, our results suggest that future studies are warranted to specifically determine whether changes in *Arc* gene and protein expression following anesthesia or other initiators of perioperative temperature loss contribute directly to the impairment of mnemonic function.

Another potentially memory altering event occuring during hypothermic anesthesia is the hyperphosphorylation of tau[19,36,37], a microtubule associated protein implicated in many neurodegenerative diseases, including Alzheimer's[38,39]. Tau hyperphosphorylation and/or aggregation is thought to contribute to memory deficits[40]. Hence, it would be interesting to dissect the respective roles of hypothermia, Arc and tau proteins in memnotic impairment following anesthesia by using tau knock-out mice.

Although it would be premature to clinically extrapolate the results of the current study to the clinical arena, the finding that anesthesia-induced hypothermia accounts for a significant change in a protein associated with memory consolidation is potentially relevant, as the perioperative occurrence of cognitive impairment[41] and hypothermia[42] following general anesthesia are quite common.

In summary, we conclude that anesthesia-induced hypothermia has profound effects on *Arc* gene expression and protein synthesis in the hippocampus, and that this effect is probably mediated through inhibition of ERK signaling. These data give novel mechanistic insights on the regulation of immediate early genes by anesthesia and hypothermia. The current study may also have significant implications for the interpretation of data from studies that have examined *Arc* activation in anesthetized animals without the control of body temperature.

Methods

Animals and treatments. 12–16-week-old Fischer 344 rats (Taconic, Germantown, NY) or 12-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were housed at 22°C, and were kept on a 12 h/12 h light/dark cycle. All animals had access to food and water *ad libitum*. The experimental protocols were approved by the Columbia University Animal Care and Use Committee or the Comité de Protection des Animaux du Centre Hospitalier de l'Université Laval and, in accordance with NIH (National Institutes of Health) and CIHR (Canadian Institutes for Health Research) guidelines, adequate measures were taken to minimize pain and discomfort.

During the isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) anesthesia studies, male rats received either isoflurane (Abbott Laboratories, Chicago, Il) at 1 MAC (1.3%) in a 30% O_2 in air mixture or a 30% O_2 in air mixture alone (control) for a period of 3 h. In the hypothermia studies, the temperature of the rats was not controlled during anesthesia and was allowed to spontaneously decrease. In the normothermia studies, rats exposed to isoflurane were anesthetized in a warmed Plexiglas anesthesia chamber equipped with a heating pad and lamps to maintain the rectal temperature at 37°C. Isoflurane and O_2 concentrations in the chamber were determined using a respiratory gas monitor (Ohmeda 5250 RGM, Datex-Ohmeda, Louisville, CO), and spontaneous ventilation was maintained throughout the entire study. The rats were killed using decapitation either immediately following isoflurane exposure (IH and IN groups for Isoflurane Hypothermia and Isoflurane Normothermia, respectively) or 24 h after (IH24 and IN24 groups for Isoflurane Hypothermia after 24 h and Isoflurane Normothermia after 24 h, respectively).

For propofol (2,6 diisopropylphenol) anesthesia, a 25 mg/ml solution of propofol (MP Biomedicals, Solon, OH) in intralipid (20% fat emulsion) was freshly prepared. Female mice were treated with either propofol (MP Biomedicals, Solon, OH) 250 mg/kg or an equivalent volume of intralipid (vehicle control for propofol, Sigma RBI, St. Louis, MO) via intraperitoneal (i.p.) injection, and anesthesia was assessed as loss of righting reflex. The dose of propofol was choosen according to our previous study[19]. One hour after injection, the mice were killed by decapitation.

Cold Water Swimming (CWS) experiments were conducted as previously described[20]. Briefly, mice swam in ice-cold water for 5 min, after which they were gently wiped dry and returned to their cages for 30 min. The temperature of the mice after CWS is ~16°C, and ~25°C 30 min. later[43]. Control animals swam for 5 min. in warm water, which keeps their temperature around 37°C[43]. Mice were killed by decapitation 30 min after CWS.

For experiments with mice lacking MEK1 or MEK2, which are the kinases upstream of ERK, we used *Mek1*−/− and *Mek2*−/− mice of either sex at 6.1 ± 2.0 month of age. *Mek2*−/− mice are straight knock-out mice[22], while *Mek1*−/− mice are conditional knockout mice that excise *Mek1* during embryonic development[24] to avoid placental lethality[23].

For all the experiments, rectal temperature was monitored with a digital thermometer (Thermalert TH-5, Physiotemp, Clifton, NJ). After decapitation, hippocampal tissue was dissected, immediately frozen on liquid nitrogen or dry ice, and then stored at −80°C. Protein extraction from frozen samples was performed as previously described[44].

All animals were handled according to procedures approved by the Columbia University Animal Care and Use Committee in accordance with National Institutes of Health (NIH) guidelines, or by the Comité de Protection des Animaux du CHUQ under the guidelines of the Canadian Council on Animal Care. Adequate measures were taken to minimize pain and discomfort.

Cell experiments. SH-SY5Y cells (ATCC #CRL-2266, American Type Culture Collection, Manassas, VA) were maintained, as recommended by the provider, in a 1 : 1 mixture of ATCC-formulated Eagle's Minimum Essential Medium and F12 Medium, in a 5% CO_2 humidified incubator at 37°C. For hypothermia exposure, cells were placed in a 5% CO_2 humidified incubator at 30°C for 2 hours, as described[19]. Cells were washed in cold phosphate buffered saline and harvested in a modified RIPA (radioimmunoprecipitation assay) buffer as described[45]. Total protein concentration was determined by the BCA (bicinchoninic acid) Protein Assay (Pierce Biotechnology, Rockford, IL), and samples were processed for Western blotting as described below.

Protein immunoblot detection. SDS-PAGE and Western blot analyses were performed as described previously[37], using the following primary antibodies: Arc mouse monoclonal clone C-7 (sc-17839, 1 : 200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or clone 40 (#612603, 1 : 1000 dilution, BD Transduction Laboratories, Franklin Lakes, NJ), anti-β-Actin monoclonal (clone AC-74, 1 : 5000 dilution, Sigma), eukaryotic elongation factor (eEF2) polyclonal (#2332, 1 : 1000 dilution, Cell Signaling Technology, Danvers, MA), phospho-eEF2 rabbit polyclonal (Thr56, #2331, 1 : 1000, Cell Signaling), ERK/MAPK rabbit polyclonal (p44/42 MAPK, #9102, 1 : 1000, Cell Signaling), and p-ERK rabbit polyclonal (Phospho-p44/42 MAPK Thr202/Tyr204, #9101, 1 : 1000, Cell Signaling). Immunoreactive bands were analyzed using a Fujifilm LAS-4000 imaging system and ImageGauge image analysis software (Version 4.2, Fujifilm USA, Valhalla, NY).

RNA extraction and real-time quantitative polymerase chain reaction analysis. Total RNA was extracted using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA). The PureLink RNA Mini Kit (Invitrogen) with on-column PureLink DNase treatment (Invitrogen) was used for the purification of the total RNA according to the manufacturer's protocol. One μg of RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR amplification was carried out in a Bio-Rad iQ5 cycler (Bio-Rad Laboratories, Hercules, CA), using 5 μL of diluted cDNA (1 : 20 dilution), 500 nM concentration of the Arc primers, 250 nM concentration of the β-actin primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μL. Arc[46] and β-actin[47] primer sequences were based on previous publications.

Statistical analysis. Group comparisons were made using one-way ANOVA with Bonferroni's *post-hoc* test, or a Student *t*-test using Prism 4 statistical analysis

software (GraphPad Software, San Diego, CA) on a MacBook Pro. All data are reported as mean ± SD, and a value of $P < 0.05$ was considered statistically significant.

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Authors contributions

Conceived and designed the experiments: R.A.W., E.P. Performed the experiments: A.B., L.V., T.O.M., F.M., C.J., N.B.E.K., F.M. Analyzed the Data: A.B., L.V., T.O.M., F.M., E.P. Contributed reagents/material/analysis tools: C.W.E., J.C. Wrote the manuscript: R.A.W., E.P. All authors reviewed the manuscript.

Additional information

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