

Intracerebellar Infusion of the Protein Kinase M zeta (PKM ζ) Inhibitor Zeta-Inhibitory Peptide (ZIP) Disrupts Eyeblink Classical Conditioning

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Protein kinase M zeta (PKM- ζ), a constitutively active N-terminal truncated form of PKC- ζ , has long been implicated in a cellular correlate of learning, long-term potentiation (LTP). Inhibition of PKM- ζ with zeta-inhibitory peptide (ZIP) has been shown in many brain structures to disrupt maintenance of AMPA receptors, irreversibly disrupting numerous forms of learning and memory that have been maintained for weeks. Delay eyeblink conditioning (EBC) is an established model for the assessment of cerebellar learning; here, we show that PKC- ζ and PKM- ζ are highly expressed in the cerebellar cortex, with highest expression found in Purkinje cell (PC) nuclei. Despite being highly expressed in the cerebellar cortex, no studies have examined how regulation of cerebellar PKM- ζ may affect cerebellar-dependent learning and memory. Given its disruption of learning in other brain structures, we hypothesized that ZIP would also disrupt delay EBC. We have shown that infusion of ZIP into the lobulus simplex of the rat cerebellar cortex can indeed significantly disrupt delay EBC.

Keywords: ZIP, PKM- ζ , eyeblink conditioning, Purkinje cell, cerebellum

Understanding the cellular mechanisms behind learning, and the subsequent formation of memory, has been a topic that has garnered scientific interest for many decades. One particular kinase that has been at the center of scientific attention in the last decade is the serine/threonine kinase protein kinase M zeta (PKM- ζ), an N-terminal truncated form of PKC- ζ that renders it constitutively active (Hernandez et al., 2003). Its role in trafficking α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors into the postsynaptic membrane is key to formation of long-term potentiation (LTP), a cellular mechanism that underlies learning and memory (Kessels et al., 2009). Sacktor and colleagues provided the first evidence that PKM- ζ has an important role in maintaining learning-related synaptic changes, by showing that interruption of PKM- ζ activity is sufficient to disrupt maintenance of hippocampal LTP (Serrano et al., 2005; Ling et al., 2006; Pastalkova et al., 2006).

The role of PKM- ζ in learning has been studied through the use of zeta inhibitory peptide (ZIP), a selective PKC- ζ pseudosubstrate inhibitor that effectively inhibits both PKC- ζ and PKM- ζ . Primarily, ZIP has been studied in relation to hippocampal-dependent memory in rats; for example, ZIP infusion into dorsal hippocam-

pus after training impaired object location retention (Hardt et al., 2010), place avoidance retention (Pastalkova et al., 2006; Serrano et al., 2008), spatial location retention in the water maze (Serrano et al., 2008), reference memory in the eight-arm radial maze (Serrano et al., 2008) and expression/retrieval of conditioned responses (CRs) in trace eyeblink conditioning (Madrónal et al., 2010). In other brain structures, including medial prefrontal cortex (mPFC), sensorimotor cortex, insular cortex, basolateral amygdala (BLA), nucleus accumbens (NAc), and dorsal striatum, ZIP has also been found to impair memory retention (Crespo et al., 2012; Evuarherhe et al., 2014; Gamiz & Gallo, 2011; He et al., 2011; Kwapis et al., 2009; Li et al., 2011; Miguez et al., 2010; Pauli et al., 2012; Shabashov et al., 2012; Shema et al., 2007; Shema et al., 2009; von Kraus et al., 2010).

Unlike other forms of learning that rely on LTP, cerebellar learning is believed to require a mechanism of long-term depression (LTD) in which AMPA receptors at parallel fiber-Purkinje cell synapses undergo endocytosis (Freeman, 2015). While no studies have investigated the role of PKM- ζ in cerebellar learning, measurement of mRNA expression of PKC- ζ and PKM- ζ using *in situ* hybridization has shown that the kinases are highly expressed in the cerebellar cortex (Oster et al., 2004). However, that study did not resolve the expression pattern of PKC- ζ and PKM- ζ with a higher level of spatial resolution at the protein level. Given the complexity of the cerebellar circuitry, we wanted to show where in the cerebellar cortex PKC- ζ and PKM- ζ are expressed. In the current study, we stained parasagittal slices of rat cerebellar cortex with a c-terminal specific α -PKC- ζ and revealed both PKC- ζ and PKM- ζ s high expression pattern throughout the cortex, primarily localized in Purkinje cell (PC) nuclei.

Delay eyeblink conditioning (EBC) is an established model for the assessment of cerebellar learning. As first elucidated by Richard F. Thompson and colleagues in the 1980s, EBC is critically dependent upon one of the deep cerebellar nuclei, the interpositus

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nucleus (IN), with modulation of learning by the cerebellar cortex (Lavond, Lincoln et al., 1984; Lavond, McCormick & Thompson, 1984, 1987; Lavond & Steinmetz, 1989; Lincoln et al., 1982; McCormick et al., 1981; McCormick & Thompson, 1984a, 1984b; Perrett et al., 1993). In EBC, an auditory stimulus, the conditioned stimulus (CS), is paired with a mild stimulation to the eye, the unconditioned stimulus (US). Learning is expressed when the subject blinks to the auditory stimulus, resulting in a CR. In the standard model for EBC, LTD at PF-PC synapses in the lobulus simplex of the cerebellar cortex leads to disinhibition of the IN which, in combination with LTP at mossy fiber-IN synapses, leads to an eyeblink CR to the CS (Thompson & Steinmetz, 2009). The IN is the sole output of the cerebellum that carries information about the eyeblink CR (Thompson & Steinmetz, 2009). In the hippocampus, LTD induction in rats has been shown to produce a down-regulation of PKM- ζ that is reversed with high-frequency afferent stimulation, suggesting that LTD may be inversely correlated with PKM- ζ expression (Hrabetova & Sacktor, 1996; Osten et al., 1996).

While the established model of cerebellar EBC has been understood to rely upon a mechanism of LTD, this model has been recently challenged; surprisingly, disrupting AMPA receptor regulation and cerebellar LTD did not impair EBC (Schonewille et al., 2011). However, PF-PC LTD may be one of a variety of plasticity mechanisms in cerebellar cortex that support cerebellar-dependent learning (Gao et al., 2012). Voltage-gated potassium channel 1.2 (Kv1.2) is an ion channel known to regulate neuronal excitability (Khavandgar et al., 2005; Southan & Robertson, 1998a, 1998b). Kv1.2 is most abundantly expressed in cerebellar basket cell (BC) axon terminals (pinneaus) and in PC dendrites (Chung et al., 2001; Koch et al., 1997; Laube et al., 1996; Wang et al., 1993). Indeed, inhibition of Kv1.2 with Tityustoxin-K α (TsTx) in cerebellar PC dendrites increases PC excitability (Khavandgar et al., 2005), while its inhibition in BC axon terminals increases inhibition of PCs (Southan & Robertson, 1998). Furthermore, we have previously shown that intracerebellar infusion of tityustoxin (TsTx), a Kv1.2 inhibitor, enhances EBC (Williams et al., 2012). Two prior studies have demonstrated that PKC- ζ associates with and is able to phosphorylate cerebellar Kv β 2 (Crocì et al., 2003; Gong et al., 1999), an auxiliary subunit that interacts with Kv1.2 (Coleman et al., 1999). Given the high levels of PKM- ζ expression in the cerebellar cortex and its possible interaction with Kv1.2, we hypothesized that ZIP would disrupt cerebellar-dependent learning.

Method

Immunohistochemistry

A 4 month old male Wistar rat (Charles River, Quebec, Canada) was euthanized according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont and cerebellar tissue was harvested. Cerebellar tissue underwent fixation with a transcardial perfusion of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde chilled to 4 °C. 400- μ m-thick parasagittal sections were cut on a vibratome and collected in PBS. Fixed cerebellar slices were briefly acetone-extracted and then incubated in blocking buffer (3% normal goat serum in PBS). Slices were then incubated for 24 hr each in primary and secondary antibodies. Primary antibodies were mouse monoclonal α -Kv1.2 (1:200) (Neuromab, Clone K14/16, Cat.

#75-008) and rabbit polyclonal α -PKC- ζ C-Terminal (1:50) (Sigma-Aldrich, Cat. #SAB4502380). The secondary antibodies were goat antimouse Alexa 488 and goat antirabbit Alexa 568 (ThermoFisher Scientific, Cat. #A-11001 and A-11036). Slices were mounted with Prolong Gold antifade with DAPI (ThermoFisher Scientific, Cat. #P-36931) and imaged using a Nikon C2 + Confocal microscope. Images were processed with ImageJ software.

EBC Subjects

Male Wistar rats (59–63 days old) were purchased from Harlan (Indianapolis, IN) or Charles River (Quebec, Canada) and housed in pairs upon arrival with access to food and water ad libitum. Rats were single housed after surgery. The colony room was maintained on a 12-hr light–dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.). Rats weighed 300–400 g before surgery. All testing took place during the light phase of the schedule and the IACUC of the University of Vermont approved all procedures.

EBC Surgery

Surgeries took place 4–6 days after arrival. Surgeries were performed under aseptic conditions. Rats were anesthetized with 3% isoflurane in oxygen. During the surgery, a 7-mm stainless steel 22-gauge guide cannula (Plastics One) was implanted into the lobulus simplex of the cerebellar cortex (AP:-11.0 from bregma; ML:-3.0; DV:-3.2; infusion at DV:-4.2).

After securing the cannula to the skull with dental cement, an 8-mm dummy cannula was placed in the guide cannula and secured with screw threads. The dummy cannula served to seal the guide cannula to prevent infection and to prevent the guide cannula from becoming obstructed before infusions. A bipolar stimulation electrode (Plastics One) was positioned subdermally immediately dorsocaudal to the ipsilateral eye. Two EMG wires for recording activity of the external muscles of the eyelid, the orbicularis oculi, were constructed from two strands of 75-mm Teflon coated stainless steel wire soldered at one end to a round threaded pedestal connector (Plastics One). The other end of the wire was passed subdermally to penetrate the skin of the upper eyelid of the eye, ipsilateral to the guide cannula. A ground wire was wrapped around two skull screws at one end and the other end was soldered to the pedestal connector. The cannula, pedestal connector, and bipolar electrode were cemented to the skull with dental cement. The wound was numbed with a local injection of 0.1 ml bupivacaine, spread out over three points around the wound. The wound was salved with antibiotic ointment (Povidone), and an analgesic (buprenorphine) and fluids (lactated Ringers) were administered (subcutaneously, s.c.) immediately after surgery. Analgesic was administered twice the following day. Rats were given 5–6 days to recover before eyeblink conditioning.

EBC Apparatus

Eyeblink conditioning took place in one of four identical testing boxes (30.5 $\times$ 24.1 $\times$ 29.2 cm; Med-Associates), each with a grid floor. The top of the box was altered so that a 25-channel tether/commutator could be mounted to it. Each testing box was kept within a separate electrically shielded, sound-attenuating chamber

(45.7 × 91.4 × 50.8 cm; BRS-LVE, Laurel, MD). A fan in each sound-attenuating chamber provided background noise of approximately 60 dB SPL. A speaker was mounted in each corner of the rear wall and a light (off during testing) was mounted in the center of the rear wall of each chamber. The sound-attenuating chambers were housed within a walk-in soundproof room.

Stimulus delivery was controlled by a computer running Spike2 software (CED). A 2.8 kHz, 80 dB tone, delivered through the left speaker of the sound-attenuating chamber, served as the CS. The CS was 365-ms in duration. A 15-ms, 4.0-mA uniphasic periorbital stimulation, delivered from a constant current stimulator (model A365D; World Precision Instruments), served as the US during conditioning. Recording of the eyelid EMG activity was controlled by a computer interfaced with a Power 1401 high-speed data acquisition unit and running Spike2 software (CED). Eyelid EMG signals were amplified (10 K) and bandpass filtered (100–1000 Hz) before being passed to the Power 1401 and from there to the computer running Spike2. Sampling rate was 2 kHz for EMG activity. The Spike2 software was used to full-wave rectify, smooth (10 ms time constant), and time shift (10 ms, to compensate for smoothing) the amplified EMG signal to facilitate behavioral data analysis.

Eyeblink Conditioning Procedure

At the beginning of each session, each rat was plugged in, via the connectors cemented to its head, to the 25-channel tether/commutator, which carried leads to and from peripheral equipment and allowed the rat to move freely within the testing box. On Day 1 (adaptation), rats were plugged in but no stimuli were delivered. They remained in the chamber for 60 min (the approximate length of a training session). Spontaneous eyelid EMG activity was sampled for the same duration and at the same time points as during the subsequent conditioning sessions (i.e., 2 s samples with an average intertrial interval (ITI) of 30 s and a range of 20–40 s). On Days 2–7 (conditioning), rats received 100 trials per day, with an average ITI of 30 s (range = 20–40 s). Each block of 10 trials consisted of the following trial sequence: 4 CS–US trials (CS preceding and coterminating with the US), 1 CS-alone trial, 4 CS–US trials, and 1 US-alone trial.

Two hours before the first session of conditioning, rats received an intracerebellar infusion of 0.50 μ l of 20 mM myristoylated-ZIP (AnaSpec, Cat. #AS-63361), 20 mM myristoylated-scrambled-ZIP (AnaSpec, Cat. #AS-63695), or phosphate-buffered saline vehicle. For infusions, the dummy cannula was removed and a 28-gauge internal cannula was inserted into the guide cannula. The internal cannula protruded 1 mm below the guide cannula tip, making the final infusion depth 4.2 mm below bregma. Infusions were made with a 10-mL Hamilton syringe loaded onto an infusion pump (KD Scientific) set to deliver 0.5 μ l of solution over 2 min. At the end of the infusion period, the internal cannula remained in place an additional 1 min to allow diffusion of the infused solution away from the cannula tip. Subsequently, the internal cannula was removed, the dummy cannula was replaced, and the rats were placed back in their home cage for 2 hr. Thereafter, the rats were plugged in, and the EBC session began. Rats were infused and tested in groups of four.

EBC Histology

Within approximately 24 hr after the final session, rats were overdosed with sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.9% saline followed by 10% buffered formalin. A small DC electrolytic lesion (100 μ A, approximately 10 s) was made by passing current through an electrode made from a 000 gauge insect pin insulated (except for 0.5 mm on the tip) with nail polish, that was placed into the guide cannula, with the tip extending out of the guide cannula by approximately 1.0 mm. The brain was removed and stored in 10% buffered formalin. Four or 5 days before sectioning, the brain was transferred to a 30% sucrose/10% buffered formalin solution. Before sectioning, cerebella were embedded in albumin–gelatin. Frozen sections of the cerebellum were taken at 60 μ m. Tissue was mounted on gelatin-coated glass slides, stained with Prussian blue (for iron deposits left by the marking lesions) and cresyl violet (for cell bodies) and cover slipped with Permount. Slides were examined under a microscope by an observer blind to group membership to confirm cannula placement.

Behavior Analysis

For the conditioning sessions, CS–US trials were subdivided into four time periods: (a) a “baseline” period, 280 ms before CS onset; (b) a nonassociative “startle” period, 0–80 ms after CS onset; (c) a “CR” period, 81–350 ms after CS onset; and (4) a “UR period,” 65–165 ms after US onset (the first 65 ms is obscured by the stimulation artifact). On CS-alone trials, the “CR” period extended for 150 ms after CS offset to capture CRs that may normally be masked by the US. For a response to be scored as a CR, eyeblinks had to exceed the mean baseline activity for that trial by 0.5 arbitrary units (where these units had a range of 0.0–5.0) during the CR period. Eyeblinks that met this threshold during the startle period were scored as startle responses and were analyzed separately. Trials in which eyeblinks exceeded 1.0 arbitrary unit during the baseline period were discarded. Comparable scoring intervals and criteria were used to evaluate spontaneous blink rate during the initial adaptation day when no stimuli were administered. The primary dependent measure for all experiments was the percentage of CRs across all CS–US (acquisition) trials of each session.

Data were analyzed using repeated-measures analysis of variances (ANOVAs) and post hoc analyses consisted of independent-samples *t* tests. We computed all statistical analyses using SPSS 23.0. A *p* value of 0.05 was set as the rejection criterion for all statistical tests.

Results

Identification of PKC- ζ /PKM- ζ Expression in Rat Cerebellar Cortex

To determine the expression of PKC- ζ and PKM- ζ in the rat cerebellar cortex, we used immunohistochemistry in parasagittal cerebellar sections. Using a Nikon C2 + Confocal microscope, we identified dense expression of PKC- ζ /PKM- ζ (red) throughout the cerebellar cortex in the molecular (ML) and granular layers (GL), as well as the pinceaus of BC axon

terminals as shown in Figure 1. The highest expression was found in the nuclei of PCs as well as throughout the ML. We used Kv1.2 (green) as a marker for BC axon terminals, which form “pincaus” around the PC soma. No expression of PKC- ζ /PKM- ζ was detected in the deep cerebellar nuclei.

PKM- ζ Function in Cerebellar Cortex Affects Cerebellar-Dependent Learning in Rats

To determine the role of endogenous PKM- ζ in cerebellar-dependent learning, we infused rats with the selective PKM- ζ pseudosubstrate inhibitor, myristoylated-ZIP (0.50 μ l; 20 nmol/ μ l; Anaspec), myristoylated-scrambled-ZIP (0.50 μ l; 20 nmol/ μ l; Anaspec), or vehicle phosphate-buffered saline (0.50 μ l). The day before infusion, rats were placed in experimental chambers and received 100 no-stimulus trials to measure spontaneous eyeblink activity. Infusions were made into the lobulus simplex of the cerebellar cortex ipsilateral to the conditioned eye 2 hr before conditioning Session 1 of six acquisition sessions of EBC. The six conditioning sessions consisted of 80 CS–US paired trials (350-ms delay paradigm), and 10 CS-alone trials and 10 US-alone trials evenly spaced within the CS–US paired trials, so that every fifth trial alternated between a CS-alone and a US-alone trial.

Before analysis, we verified all cannula placements to ensure placement was in the lobulus simplex of the cerebellum ipsilateral to the conditioned eye. In total, 31 rats (13 ZIP; 10 Scr-ZIP, 8 Veh) were included in the analysis. Three rats were removed before data analysis because of poor EMG signals ($n = 2$) or illness ($n = 1$). None were removed because of incorrect cannula placement.

As shown in Figure 2, the results suggest that although all three groups learned, the vehicle-infused group exhibited significantly

more eyeblink CRs than the ZIP-infused group. A 3 (group: ZIP; Scr-ZIP; Veh) $\times$ 6 (session) repeated-measures ANOVA on the percentage of CRs confirmed this. A main effect of session, $F(5, 140) = 36.30$, $p < .05$ and a Group $\times$ Session interaction, $F(10, 140) = 1.99$, $p < .05$ were revealed. The main effect of group approached, but did not attain, statistical significance ($p = .067$). Post hoc independent-samples t tests of the significant interaction revealed that the percentage of CRs in Group ZIP were significantly lower than Group Veh in Sessions 2 ($p = .008$), 3 ($p = .016$), 4 ($p = .042$), and 6 ($p = .016$). The same analyses comparing Group Scr-ZIP to Group Veh revealed that the percentage of CRs did not differ significantly ($ps > 0.08$); however, Group Scr-ZIP showed a greater percentage of CRs than Group ZIP in Sessions 5 ($p = .020$) and 6 ($p = .021$).

To confirm that intracerebellar infusions of ZIP or Scr-ZIP before acquisition Session 1 did not change the reflexive startle response to the CS, we analyzed the percentage of startle responses to the CS (eyeblinks with an onset latency of 80 ms or less after CS onset). A 3 (group: ZIP; Scr-ZIP; Veh) $\times$ 6 (session) repeated-measures ANOVA yielded no significant effects ($F_s < 1.2$, $ps > 0.34$). To confirm that intracerebellar infusions of ZIP or Scr-ZIP before acquisition Session 1 did not change the reflexive eyeblink to the US, we analyzed the amplitude of URs on US-alone trials. A 3 (group: ZIP; Scr-ZIP; Veh) $\times$ 6 (session) repeated-measures ANOVA also yielded no significant effects ($F_s < 0.9$, $ps > 0.47$). Thus, infusion-related changes in reflexive responding to either the CS or the US are unlikely to explain our results. Finally, intracerebellar infusions of ZIP or Scr-ZIP did not affect measures of the learned response itself. This was confirmed for measures of CR topography from CS-US trials (CR amplitude; CR onset latency)

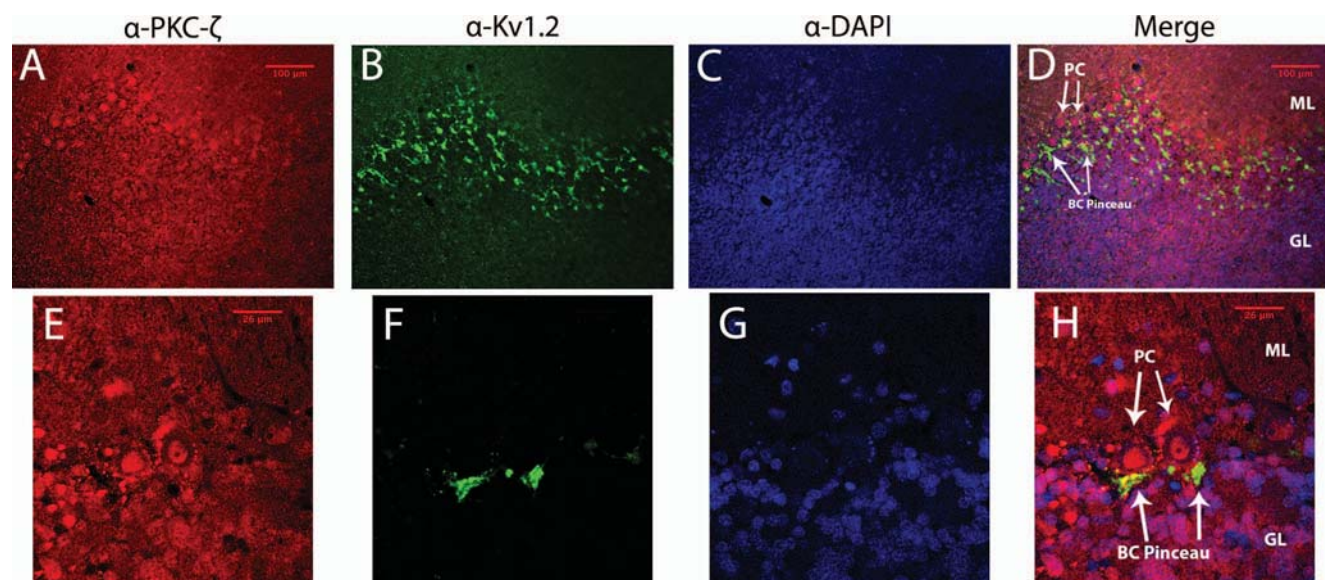


Figure 1. Expression of protein kinase C zeta (PKC- ζ) and Kv1.2 in cerebellar cortex. (A and E) Overnight incubation of fixed rat cerebellar slices with α -PKC- ζ (red) gives positive signal throughout the cortex in the molecular (ML) and granular layers (GL) as well as the pincaus of basket cell (BC) axon terminals. Its highest expression was found in the nuclei of Purkinje cells (PC) and throughout the molecular layer. (B and F) Overnight incubation with α -Kv1.2 (green) gives positive signal throughout cortex with highest expression in the pincaus of BC axon terminals. (C and G) Blue signal is indicative of DAPI nuclear stain. Merged channels are shown in D and H. See the online article for the color version of this figure.

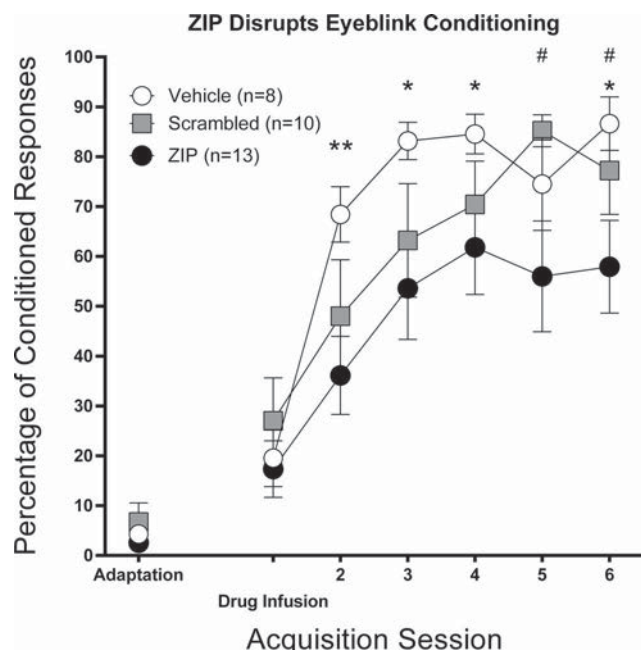


Figure 2. Infusion of zeta-inhibitory peptide (ZIP) into the lobulus simplex of the cerebellar cortex significantly disrupts eyeblink conditioning (EBC). Error bars indicate *SEM*. Rats received either 0.5 μ l intracerebellar infusion of ZIP (20 nmol/ μ l) or Scr-ZIP (20 nmol/ μ l) or PBS vehicle before the first session of acquisition (80 CS–US [conditioned stimulus–unconditioned stimulus] trials per session with 350 ms delay, with interspersed CS and US probe trials). A 3 (group) $\times$ 6 (session) repeated-measures analysis of variance (ANOVA) on the percentage of CRs on CS–US trials revealed a significant main effect of session, $F(5, 140) = 36.30$, $p < .05$ and a Group $\times$ Session interaction, $F(10, 140) = 1.99$, $p < .05$. The main effect of group approached, but did not attain, statistical significance ($p = .067$). Follow-up independent-samples *t* tests of the significant interaction revealed that the percentage of CRs in Group ZIP were significantly lower than Group Veh in Sessions 2 ($p = .008$), 3 ($p = .016$), 4 ($p = .042$), and 6 ($p = .016$). The same analyses comparing Group Scr-ZIP to Group Veh revealed that the percentage of CRs did not differ significantly ($ps > 0.08$); however, Group Scr-ZIP showed a greater percentage of CRs than Group ZIP in Sessions 5 ($p = .020$) and 6 ($p = .021$). Asterisks (*) and ** indicate $p < .05$ and $p < .01$, respectively, for Group ZIP versus Group Veh. Pound sign (#) indicates $p < .05$ for Group Scr-ZIP versus Group ZIP.

and from CS-alone trials (CR peak latency) with a series of 3 (group: ZIP; Scr-ZIP; Veh) $\times$ 6 (session) repeated-measures ANOVAs. For these analyses, rats without any CRs in one or more sessions were removed from the analysis, because CR topography values are undefined in these cases. A significant effect of session was revealed for CR amplitude, $F(5, 95) = 12.44$, $p < .01$ and for CR peak latency, $F(5, 90) = 4.81$, $p = .001$; the session effect for CR onset latency approached, but did not attain, statistical significance, $F(5, 95) = 2.25$, $p = .056$. No other effects were significant ($Fs < 1.6$, $ps > 0.23$).

Histological Analysis

Figure 3 shows approximate locations of confirmed cannula tip placements in the lobulus simplex of cerebellar cortex. Cannula tip

placements of rats included in the analyses were confirmed to be within the lobulus simplex of the cerebellar cortex. Cannula tip placements were slightly more dorsal than the cannula tip placements reported in rats to be the most effective for impairment of EBC by muscimol infusion (Steinmetz & Freeman, 2014). More important, however, our placements were clearly distant from the deep cerebellar nuclei.

Discussion

We have shown that a single infusion of the PKM- ζ inhibitor, ZIP, into the lobulus simplex of the cerebellar cortex, 2 hr before the first acquisition session, impairs EBC. This is the first demonstration that PKM- ζ may be important for cerebellar-dependent memory, and is consistent with the established literature showing ZIP-mediated impairment of other forms of learning and memory (e.g., object location, spatial location, radial-maze learning, and place avoidance) mediated by other brain structures (e.g., dorsal hippocampus).

One interpretation of these results is that knocking out the gene for PKC- ζ /PKM- ζ causes compensatory, abnormal upregulation,

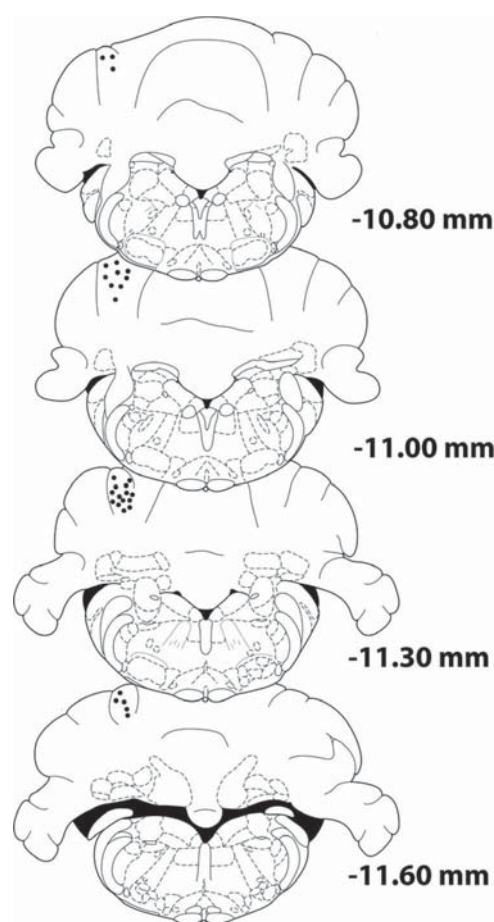


Figure 3. Cannula placements in lobulus simplex of the cerebellar cortex. Numbers on the right are millimeters posterior from bregma. From *The Rat Brain in Stereotaxic Coordinates* (4th ed.), pp. 226–229 by G. Paxinos & C. Watson, 1998, New York, NY: Academic Press. Copyright, 1998 by Elsevier Academic Press. Adapted with permission.

or enhanced phosphorylation, of a closely related PKC isoform, PKC- ι , and that ZIP also blocks the activity of this kinase (Jalil et al., 2015; Lee et al., 2013; Selbie et al., 1993). In addition, others have shown that PKM- ζ conditional knockout mice show disrupted late-phase LTP, and that infusion of PKM- ζ antisense oligonucleotides into the hippocampus could also function similar to ZIP (Tsokas et al., 2012). Late-phase LTP is a protein synthesis-dependent form of LTP that is understood to be a key component of long-term memory (Abel et al., 1997; Frey et al., 1988). Furthermore, it is important to note that PKM- ζ has been shown to play a role in memory using techniques other than ZIP infusion; Sacktor and colleagues have shown that PKM- ζ lentiviral overexpression in rat insular cortex 5 days before acquisition enhances CTA, while lentiviral overexpression of dominant-negative PKM- ζ 5 days before acquisition disrupts CTA (Shema et al., 2011).

While the specificity of ZIP is debated, it remains the most specific inhibitor for PKC- ζ /PKM- ζ . However, another concern with ZIP is its use with a proper control; several studies using Scr-ZIP have shown that it behaves similar to ZIP and could also disrupt both LTP and memory. In one study, Scr-ZIP was found to be equally effective as ZIP at reversing LTP in mouse hippocampal slices (Volk et al., 2013). This same study also found that ZIP and Scr-ZIP can both inhibit purified PKC- ζ and PKM- ζ and had overlapping inhibition curves. To rule out an effect because of the myristoylation group present on both Scr-ZIP and ZIP, Volk and colleagues demonstrated that myr-PKI, a PKA inhibitor, was without effect and not responsible for disrupting LTP maintenance. In another study examining the BLA, rats that received Scr-ZIP infusions into the BLA showed a slight, but nonsignificant, decrease in freezing behavior compared with the saline controls (Kwapis et al., 2009). The authors suggested that Scr-ZIP may weakly bind and inactivate some PKM- ζ because of its nearly palindromic basic sequence, and recommended use of vehicle as the proper control (Kwapis et al., 2009). Using an in vitro kinase assay, Lee and colleagues demonstrated that in fact both Scr-ZIP and ZIP inhibit PKM- ζ and PKC- ζ with only a 7.3-fold difference in their K_i values (Lee et al., 2013).

In defense of Scr-ZIP, Yao and colleagues recently demonstrated in hippocampal slices that a 5 μ M concentration of Scr-ZIP had no effect on PKM- ζ -mediated potentiation of post-synaptic CA1 pyramidal cell AMPA receptor responses (Yao et al., 2013). However, they did acknowledge and demonstrate that in higher doses, Scr-ZIP inhibited PKM- ζ and, therefore, may not be a proper control. This poses potential problems with its use in behavioral studies, as the concentration of initial drug infusion must be greater than the expected target dose, to account for loss of the drug during diffusion. In a letter to the editor of *Hippocampus*, Sacktor and Fenton addressed the concerns surrounding the high dosage of ZIP, but not Scr-ZIP, in hippocampal infusions, by using immunocytochemistry to show biotin-labeled ZIP diffusion (Sacktor & Fenton, 2012). However, it remains to be shown what the final deliverable concentration of ZIP would be in behavioral infusions, especially in other brain structures. Given that Scr-ZIP can inhibit PKM- ζ , we suggest caution in interpreting results of experiments that use Scr-ZIP as the only control for ZIP in behavioral infusion studies.

To explain our ZIP-mediated disruption of EBC, we suggest three possible models. One well-studied mechanism of EBC in-

volves LTD at PF-PC synapses; cerebellar PKM- ζ may induce endocytosis of these AMPA receptors rather than maintain them on the surface, and thus, may maintain LTD as opposed to LTP. It is believed that LTD at PF-PC synapses in the cerebellar lobulus simplex during EBC leads to disinhibition of the IN, the sole output of cerebellum that carries information about the eyeblink CR (Thompson & Steinmetz, 2009). This would suggest that PKM- ζ acts to enhance cerebellar-dependent learning, similar to its function in other brain structures. Therefore, infusion of ZIP may prevent LTD facilitation and lead to an increase in PC AMPA receptors at the PF-PC synapse, resulting in disruption of EBC. However, Schonewille and colleagues have also shown that disrupting AMPA receptor endocytosis and cerebellar LTD does not impair EBC (Schonewille et al., 2011).

While LTD in the cerebellar cortex is a well-studied mechanism of EBC, recent work suggests that LTP in the cerebellar cortex may play a more important role in cerebellar learning than previously thought (Grasselli & Hansel, 2014). To study the role of LTP in EBC, Schonewille and colleagues generated mice with knockouts of calcium/calmodulin-activated protein-phosphatase-2B (PP2B) that abolished LTP in PCs and their ability to increase their intrinsic excitability (Schonewille et al., 2010). These mutant mice demonstrated impaired acquisition of delay EBC and impaired adaptation of vestibular-ocular reflex; therefore, it is possible that PKM- ζ disruption by ZIP may impair PF-PC LTP maintenance as it does at CA3-CA1 synapses in the hippocampus by affecting the ability of PKM- ζ to maintain GluR2-AMPA receptors at the synapse (cf. Hansel, 2005).

Our prior work has provided evidence for another mechanism by which EBC may occur, through modulation of voltage-gated potassium channels, particularly Kv1.2. We have shown that intracerebellar infusion of both tityustoxin (TsTx), a Kv1.2 inhibitor, and secretin, an endogenous Kv1.2 suppressor, enhances EBC (Williams et al., 2012). Furthermore, we have demonstrated that the secretin receptor antagonist, 5–27 secretin, disrupts EBC (Fuchs et al., 2014). Kv1.2 is most abundantly expressed in cerebellar BC axon terminals (pinceaus) and in PC dendrites (Chung et al., 2001; Koch et al., 1997; Laube et al., 1996; Wang et al., 1993). Through its ability to alter neuronal excitability (Khavandgar et al., 2005; Southan & Robertson, 1998), Kv1.2 is in a unique position to ultimately affect the excitability of PCs and, thus, be able to modulate learning through mechanisms other than LTD/LTP. Of interest to the authors, our preliminary data in HEK 293 cells show that PKM- ζ induces a significant decrease in Kv1.2 surface expression (Chihabi et al., 2015). Thus, we propose that the current results may at least be partially explained by disruption of a PKM- ζ induced decrease in Kv1.2 surface expression in cerebellar cortex during EBC.

Furthermore, we have shown that PKC- ζ /PKM- ζ is densely expressed throughout the cerebellar cortex with the highest expression in PC nuclei. We did not detect PKC- ζ /PKM- ζ in the deep cerebellar nuclei, including the IN. Thus, PKM- ζ may be involved in EBC only through modulation of the cerebellar cortex. This finding extends a previous experiment by Oster and colleagues in which PKC- ζ /PKM- ζ mRNA expression was found only in the cerebellar cortex (Oster et al., 2004). These results, together with the fact that our cannula placements were fairly dorsal to the IN, strongly suggest that the impairment in EBC produced by ZIP

infusion in the current study was because of effects on the cerebellar cortex.

It is important to note that our study is one of a few to look at ZIP's involvement in early acquisition of memory. Our reasoning for beginning with this time point for infusion comes from our previous data showing that blocking Kv1.2 in cerebellar cortex in the first session of EBC can facilitate learning (Williams et al., 2012) and from data showing that PKM- ζ can modulate cell surface expression of Kv1.2 (Chihabi et al., 2015). Our infusion of ZIP 2 hr before the first acquisition session is different from most studies that looked at the effect of ZIP after learning has already occurred. Notably, in our experiment the impairment in EBC produced by infusion of ZIP into the cerebellar cortex persisted through Days 2–6 despite the fact that ZIP infusion occurred only before Session 1. However, this is consistent with what is known about PKM- ζ ; its temporary ZIP-mediated disruption causes permanent memory loss and learning deficits (Sacktor, 2011). Thus, ZIP infusion into cerebellar cortex before Day 1 may have prevented consolidation of EBC from that first day of acquisition, effectively putting these rats 1 day behind the vehicle control rats in acquisition. The lower asymptote attained by Day 4 of acquisition, after just a single infusion of ZIP before Day 1 of acquisition, is more difficult to explain. A lower asymptote seems more similar to the effects of a lesion of the cerebellar cortex at the base of the primary fissure (Steinmetz & Freeman, 2014). That study used a very similar delay EBC procedure to our procedure. However, we also observed a much faster rate of learning compared with those rats.

Of interest to the authors, one study that looked at associative recognition memory found that ZIP disrupts memory in both the hippocampus and mPFC when infused postacquisition; however, ZIP infusion preacquisition disrupted memory in the mPFC but not the hippocampus (Evuarherhe et al., 2014). Evuarherhe and colleagues postulated a possible role for the GluR2 subunit of AMPA receptors in this temporal difference; they found that blocking GluR2-dependent removal of AMPA receptors through the coinfusion of peptide GluR2_{3Y} did not affect the impairment caused by ZIP in the mPFC, but did disrupt the impairment of ZIP in the hippocampus. Trafficking of GluR2 is critical for maintenance of hippocampal late-phase LTP (Yao et al., 2008) and past studies have shown that blocking endocytosis of GluR2 is sufficient to prevent impairments in LTP maintenance caused by ZIP (Migues et al., 2010). This suggested that impairment by ZIP in the mPFC was independent of GluR2 receptors, indicating an alternative pathway in PKM- ζ dependent mPFC memory formation (Evuarherhe et al., 2014). One possibility in the cerebellum is regulation of Kv1.2 by PKM- ζ . In conclusion, we have shown that PKC- ζ and PKM- ζ are highly expressed in the cerebellar cortex and that intracerebellar infusion of a PKM- ζ inhibitor, ZIP, before Session 1 of conditioning disrupts cerebellar-dependent delay EBC. We propose that the effect of ZIP on cerebellar EBC may be through disruption of both AMPA receptor and Kv1.2 channel maintenance in the cerebellar cortex.

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