Research

Lifelong reductions of PKMζ in ventral hippocampus of nonhuman primates exposed to early-life adversity due to unpredictable maternal care

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Protein kinase Mζ (PKMζ) maintains long-term potentiation (LTP) and long-term memory through persistent increases in kinase expression. Early-life adversity is a precursor to adult mood and anxiety disorders, in part, through persistent disruption of emotional memory throughout life. Here we subjected 10- to 16-wk-old male bonnet macaques to adversity by a maternal variable-foraging demand paradigm. We then examined PKMζ expression in their ventral hippocampi as 7- to 12-yr-old adults. Quantitative immunohistochemistry reveals decreased PKMζ in dentate gyrus, CA1, and subiculum of subjects who had experienced early-life adversity due to the unpredictability of maternal care. Adult animals with persistent decrements of PKMζ in ventral hippocampus express timid rather than confrontational responses to a human intruder. Persistent down-regulation of PKMζ in the ventral hippocampus might reduce the capacity for emotional memory maintenance and contribute to the long-lasting emotional effects of early-life adversity.

Early-life adversity is associated with an increased vulnerability to stress-related disorders that is maintained into adulthood, suggesting a very long-lived effect on emotional memory by the early-life event (Coplan et al. 1996). Although several structural and neurochemical sequelae of early-life adversity have been reported (Teicher et al. 2003; Jackowski et al. 2011), the direct effects of early-life adversity on the molecular substrates maintaining long-term memory storage have not been explored.

Accumulating evidence supports a crucial role for the autonomously active, atypical protein kinase C (PKC) isoform protein kinase Mζ (PKMζ) in maintaining synaptic long-term potentiation (LTP), a putative physical substrate for memory, and long-term memory storage (Ling et al. 2002; Pastalkova et al. 2006; Glanzman 2013; Sacktor and Fenton 2018). The autonomous activity of PKMζ is due to its unusual structure that differs from other PKC isoforms (Sacktor et al. 1993). Most PKCs consist of two domains: a catalytic domain and an autoinhibitory regulatory domain that suppresses the catalytic domain. Therefore, most PKCs are inactive until second messengers bind to the regulatory domain and induce a conformational change that releases the autoinhibition. Because second messengers that activate PKCs such as Ca^{2+} or diacylglycerol have short half-lives, most PKCs are only transiently activated.

PKMζ, in contrast, consists of an independent PKCζ catalytic domain, and the absence of an autoinhibitory regulatory domain results in autonomous and thus persistent activity once the kinase is synthesized. PKMζ mRNA is transcribed from an internal promoter within the PKCζ/PKMζ gene that is active only in neural tissue (Hernandez et al. 2003). The mRNA is translationally repressed and transported to dendrites of neurons (Muslimov et al. 2004). High-frequency afferent synaptic activity during LTP induction or learning derepresses PKMζ mRNA translation, triggering new synthesis of PKMζ protein (Osten et al. 1996; Hernandez et al. 2003; Tsokas et al. 2016; Hsieh et al. 2017).

Once increased, the steady-state amount of PKMζ remains elevated during LTP or long-term memory maintenance. Recent work with quantitative immunohistochemistry (IHC) shows that spatial conditioning induces persistent increases of PKMζ in somatic and selective dendritic compartments of dorsal hippocampal CA1 pyramidal cells that can last at least 1 mo (Hsieh et al. 2021). The persistent increases are preferentially expressed in CA1 pyramidal cells that were activated during the formation of the memory, specifically at the termination zone of the Schaffer collateral/commissural inputs from subfield CA3. In contrast, persistent PKMζ increases are not evident in stratum lacunosum-moleculare, the termination zone that originates in entorhinal cortex that nonetheless is capable of expressing PKMζ. Postsynaptic domain-specific PKMζ expression patterns hint at distinct circuit-specific modifications of cortical–hippocampal synaptic function by maturational and experiential factors.

Persistent changes in PKMζ expression are also associated with changes in the capacity for learning and memory across the

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life span of animals. Decreased memory ability in aged rats is associated with decreased training-induced, persistent PKMζ expression in prelimbic cortex, and increases in PKMζ are crucial for the cognition-enhancing effects of environmental enrichment in the aged animals (Chen et al. 2016). Hara et al. extended the connection between PKMζ and cognitive function to nonhuman primates (NHPs), showing that levels of PKMζ expression in dentate gyrus (DG) axospinous synapses correlate with successful performance on cognitive tasks in young and aged monkeys (Hara et al. 2012). These studies suggest that persistent down-regulation of PKMζ may comprise an important pathophysiological mechanism for cognitive impairment.

Here we used a validated NHP model of early-life adversity, maternal variable-foraging demand (VFD), to explore the links between adversity in infancy and PKMζ expression in adulthood (Coplan et al. 1996; Jackowski et al. 2011). Previous studies of the VFD paradigm have revealed that both infants and their mothers exposed to VFD show significant cerebrospinal fluid (CSF) elevations of the stress neuropeptide, corticotropin-releasing factor (CRF). Moreover, the magnitude of CRF change in mothers and infants are positively correlated, suggesting synchronization of maternal–infant stress responses to the VFD stressor (Coplan et al. 2005). From a behavioral standpoint, maternal social rank plays a negligible role in determining an aggregate score of maternal–infant proximity, suggesting preferential attention of mothers to their infants. During the VFD condition, maternal social rank predicts >80% of the variance of maternal–infant proximity, suggesting mothering patterns are interrupted by preferential orientations to social rank; the latter determines food accessibility (Coplan et al. 2015). Dominant females show relative increases in maternal–infant proximity, whereas subordinate females show relative reductions in maternal–infant proximity. Neither pattern of attachment ameliorates an abnormal association between CSF oxytocin concentrations and hypothalamic-pituitary-adrenal (HPA) axis activity (Coplan et al. 2015). Offspring exposed to VFD rearing assessed both as juveniles and as full adults demonstrate persistent increases in CSF CRF concentrations in comparison with controls reared under non-VFD conditions (Coplan et al. 1996, 2001).

Our prior neurohistological studies pointed to the DG as a region particularly vulnerable to VFD exposure, as shown by reduced trophic signaling and neurogenesis (Jackowski et al. 2011; Perera et al. 2011; Schoenfeld et al. 2021). We therefore hypothesized that early-life adversity due to unpredictable maternal care (for brevity, subsequently referred to as "early-life adversity") reduces the persistent expression of PKMζ within the DG of ventral intrahippocampal neurocircuitry that mediates affective memory processing (Fanselow and Dong 2010). We used PKMζ antisera validated by the lack of immunostaining in PKMζ-null mice (Hsieh et al. 2021) to examine PKMζ expression in ventral hippocampus (NHP anterior hippocampus) in both DG granule cell layer and the stratum moleculare of the suprapyramidal blade that receives direct input from entorhinal cortex, as well as other regions encompassing the hippocampal formation, including the hilus, CA3, CA1, and subiculum.

To assess behavioral correlates of hippocampal PKMζ expression, we used a stress-inducing paradigm designed specifically for singly housed bonnet macaque male NHPs, which we refer to as the "human exposure response" (Jackowski et al. 2011; Hamel et al. 2017), which is a variation of the paradigm used in human exposure studies by Kalin et al. in rhesus macaques (Kalin and Shelton 1989). On exposure to a direct human presence, singly housed adult male bonnet macaques react with a dichotomy of responses—confrontational versus timid (see the Materials and Methods) (Jackowski et al. 2011). In our macaque colony, groups of fully adult males are necessarily housed individually to prevent injury sustained during male agonistic encounters, whereas adult females and/or juveniles are safely housed in social groups. Because group housing of nursing females and/or juveniles of both sexes elicits a range of behaviors intrinsic to the species' social repertoire (Rosenblum et al. 2001; Coplan et al. 2015) that complicates behavioral analyses to human exposure, we restricted our current study to male macaques.

Results

Early-life adversity induces lifelong down-regulation of PKMζ in ventral hippocampus

On inspection, the expression of PKMζ is deficient in the ventral hippocampus of adult male animals who had experienced a 16-wk period of early-life adversity due to VFD initiated when they were 10- to 16-wk-old infants, as compared with control non-VFD subjects (Figs. 1 and 2). The reduction observed by PKMζ immunofluorescence (Fig. 1A) is corroborated by PKMζ immunohistochemistry using peroxidase staining, which reveals prominent decreases of PKMζ labeling in pyramidal cell bodies and dendrites (Fig. 2). Comparing animals who experienced VFD ($n=6$) and controls ($n=4$), the mean PKMζ immunofluorescence signal intensities in six ventral hippocampal formation subregions across two groups show a significant interaction of VFD and subregion ($F_{(5,40)}=6.30$, $P=0.0002$, $\eta_p^2=0.44$; note effect size is at least threefold larger than stipulated by Cohen [$\eta_p^2 \geq 0.14$ denotes a large effect size; Cohen 1973]), and a main effect of hippocampal subregion ($F_{(5,40)}=17.69$, $P<0.0001$, $\eta_p^2=0.69$), but not a main effect of VFD ($F_{(1,8)}=4.27$, $P=0.07$, $\eta_p^2=0.35$). The planned comparisons for region reveal a significant rearing group effect in three hippocampal subregions: stratum moleculare of the DG, CA1, and subiculum, and no identifiable differences in the granule cell layer of DG, hilus, or CA3 (Fig. 1B).

Human exposure response

On exposure to a human stranger, macaques react with either species-specific confrontational behavior, or with behavior characterized by continual or intermittent timidity (Jackowski et al. 2011; Hamel et al. 2017). As vulnerability to environmental stress is a key sequela of early-life adversity leading to decreased hippocampal function (Coplan et al. 1996; Jackowski et al. 2011), we predict that timid responses to an intruder that had been measured antemortem would be associated with reductions in hippocampal PKMζ expression. The results reveal that subjects displaying timid responses show a main effect of decreased hippocampal PKMζ expression ($n=6$), compared with animals exhibiting confrontational responses ($n=3$) ($F_{(1,7)}=6.26$, $P=0.04$, $\eta_p^2=0.47$) (Table 1). In addition, there is a main effect of hippocampal subregion ($F_{(5,35)}=10.44$, $P<0.0001$, $\eta_p^2=0.60$), but no interaction between human intruder response and hippocampal subregion ($F_{(5,35)}=2.41$, $P=0.06$, $\eta_p^2=0.26$). The planned comparisons for region reveal subjects with timid responses express less PKMζ in CA3 and the stratum moleculare and granule cell layer of DG.

Discussion

Here we present the first evidence that early-life adversity during infancy produces deficits of PKMζ expression in ventral hippocampus of adult NHPs. Limitations of the study include the relatively small number of subjects in line with other studies of NHPs. In addition, although VFD is carried out in social pens of maternal–infant dyads exposed to unpredictable foraging, grown VFD and non-VFD males are singly housed for their safety to avoid dangerous agonistic encounters. Single housing is an unavoidable limitation for measuring social behavior because socially housed adult

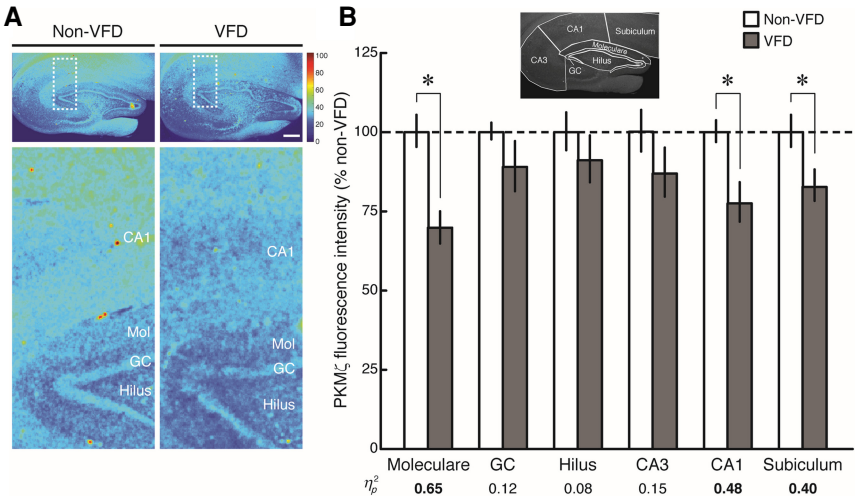

Figure 1. Persistent decreases of protein kinase Mζ (PKMζ) expression in ventral hippocampal subregions of adult nonhuman primate (NHP) subjects reared as infants under conditions of variable-foraging demand (VFD), compared with adult, non-VFD-reared controls. (*A*) Representative PKMζ immunocytochemistry shows that PKMζ decreases in the ventral hippocampus following VFD. (*Top*) Confocal images of ventral hippocampus. White rectangles indicate regions shown in detail *below*. Color-coded scale bar at *right*. (*Bottom*) Representative region of interest (ROI): CA1, (Mol) stratum moleculare of the suprapyramidal blade of the dentate gyrus (DG), (GC) granule cell layer of the suprapyramidal blade of the DG, Hilus. Scale bar in *top right* panel, 500 µm in *top* panels and 80 µm in *bottom* panels. (*B*) Mean ± SEM of PKMζ immunointensity in VFD-reared animals ($n = 6$), compared with non-VFD controls ($n = 4$, set at 100%). (*Insert*) Diagram outlining ROIs in the ventral hippocampus of bonnet macaque. Significant differences, denoted by an asterisk, are for DG stratum moleculare, CA1, and subiculum. Effect sizes (partial η_p^2 is indicated on the *X*-axis) for the subregions with significantly different *P*-values are shown in bold *below*.

males will contest dominance with the harem male, with the likelihood of severe wounding. Another limitation is the lack of inclusion of female subjects, which is a consequence of the complexity of measuring behavioral responses to human exposure due to normative expression of the species-specific social repertoire intrinsic to social housing. Unpublished data (JD Coplan) indicate that group-housed females exhibit a role for ventral hippocampal PKMζ in the maintenance of social affiliation.

Because persistent changes in the amount of PKMζ are thought to maintain experience-dependent information in the circuitry of the brain (Hsieh et al. 2017, 2021; Gao et al. 2018), the reduction of PKMζ associated with early-life adversity might be due to ongoing, lifelong alterations in the induction of synaptic plasticity in ventral hippocampal circuitry. Thus, early-life adversity could cause long-lasting suppression of the induction of LTP that persistently increases PKMζ expression, and/or long-lasting augmentation of the induction of long-term depression that persistently decreases PKMζ (Hrabetova and Sacktor 1996). This notion is in line with other sequelae of early-life adversity in NHP, including decreases in neurogenesis in DG in which the newborn neurons may be more sensitive to LTP induction compared with mature neurons (Snyder et al. 2001; Toda and Gage 2018). Future studies will be required to determine the interactions between VFD and stress-related changes in neuromodulatory signaling or neural circuitry that may alter the induction of synaptic plasticity. In addition to changes in induction, epigenetic modification, such as hypermethylation of the PKMζ promoter that reduces PKMζ gene expression (Chen et al. 2016), is a potential mechanism for the long-lasting down-regulation in PKMζ expression after early-life adversity.

The reductions of PKMζ in adulthood following early-life adversity are selective to suprapyramidal DG stratum moleculare, which receives input to the hippocampal formation from entorhinal cortex, and the hippocampal formation output subregions, CA1 and subiculum (Fig. 3). Although it is tempting to think of the entorhinal cortex → DG → CA3 → CA1 → subiculum pathway as a feedforward sequence of relays, these connections form a network of nested short and long loops because each subfield receives multiple inputs. CA1, for example, receives both an input from CA3 and a distinct direct input from the entorhinal cortex (Fig. 3; Kajiwara et al. 2008). Moreover, electrophysiological studies demonstrate that each subfield is strongly controlled by, and can maintain its own self-organizing activity due to local inhibitory networks (Colgin et al. 2009; Dvorak et al. 2018). Thus, the reduction of the molecular substrate of LTP maintenance preferentially at the postsynaptic sites of entorhinal inputs to DG, CA1, and at the inputs to subiculum might functionally weaken these projections. We speculate that this weakening of inputs of sensory information might bias hippocampal processing in favor of local self-originating activity at the expense of extrinsic entorhinal-originating activity. Such a VFD-induced bias might promote self-referential information processing that resembles rumination, which is in line with the muted behavioral responses we have observed in socially housed juvenile VFD subjects in response to a human intruder donning a clown face (Rosenblum et al. 2001).

Notably, the regions that show preferential lifelong reductions of PKMζ in ventral hippocampus after early-life adversity—CA1 and suprapyramidal DG stratum moleculare—are also regions that show preferential persistent increases in dorsal hippocampus after spatial memory conditioning, as well as the layer 3 entorhinal terminals in CA1 stratum lacunosum-moleculare, which do not change with the conditioning (Hsieh et al. 2021). Thus, the hippocampal regions that selectively store long-term information under normative conditions by LTP maintenance through increases in PKMζ expression in dorsal hippocampus are the ones that show reductions of PKMζ after early-life adversity in ventral hippocampus. Whereas the dorsal/posterior hippocampus is critical to spatial navigation (Ekstrom et al. 2003), the ventral/anterior hippocampus may serve as a neural substrate facilitating "emotional navigation" of both the internal and external environment (Fanselow and Dong 2010; Kheirbek et al. 2013). VFD deficits in behavioral "navigation" are evident (Rosenblum et al. 2001; Tavares et al. 2015), in line with the increase in timidity that we observed in animals with decreased ventral hippocampal PKMζ.

VFD rearing is a contributing factor to timid responses to human exposure seen in adulthood, and the sites of down-regulation in PKMζ associated with VFD rearing and with timid behavioral responses partially overlap. The VFD group shows selective PKMζ down-regulation in input and output regions of the hippocampal complex, whereas animals expressing timid responses show general decreases in the hippocampus, particularly CA3 and DG. Of the subregions examined, the stratum moleculare of DG shows the numerically largest PKMζ decreases and large effect sizes both after VFD (Fig. 1) and in association with timidity (Table 1), and therefore could be a key region by which early-life adversity contributes to the timidity observed in response to human exposure. Survival of an organism enduring stressful environments may, under

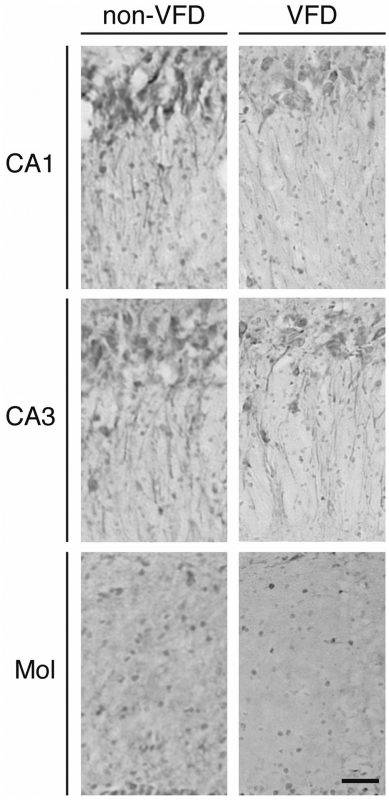

Figure 2. Protein kinase Mζ (PKMζ) expression in variable-foraging demand (VFD) and non-VFD subjects detected by immunohistochemistry using peroxidase staining. Representative images show reduction of PKMζ immunostaining in CA1 and suprapyramidal dentate gyrus (DG) stratum moleculare (Mol), as well as a modest change in CA3 in the VFD subject, compared with non-VFD. Pyramidal cell layer is at the *top* and stratum radiatum at the *bottom* of CA1 and CA3 images; border of granule cell layer is at the *bottom* of stratum moleculare images. Scale bar, 20 µm.

certain scenarios, be adaptively best served through diminution of "emotional navigation." Impairment of emotional navigation may lead to uncertainty, timidity, fear, avoidance, and even immobilization during which time imminent threats to survival may dissipate or even abate.

It is of interest, in psychodynamic terms, that the VFD condition occurs at a time in macaque infancy that would correspond to a human age of childhood "amnesia" (<3 yr) prior to the recall of "personal memories," which evidently requires the formation of explicit memories that can be encoded for long-term storage and retrieval (Bruce et al. 2000). We postulate that the early adversity induced by unpredictable maternal care implicit to VFD rearing persistently impacts the faculty of "affective memory," a form of nondeclarative or implicit memory (Reber 2013). We speculate that affective memory may act as an implicit regulator of trait by subicular outputs that include nucleus accumbens, amygdala, and prefrontal cortex (Burns et al. 1996). Affective memory potentially underlies components of personal identity (Karavanta 2013) and might modulate risk for the development of psychiatric disease, including posttraumatic stress disorder (PTSD) and depression (Bryant et al. 2007; van Vugt et al. 2012). An impairment of affective memory through reduction of PKMζ expression might also serve a protective or defensive role through reduction of the putative caloric demand required for the maintenance of memories of uncertainty and stress, including those formed early in infancy in the context of adversity (Coplan et al. 2018). Although we are unaware of published work on PKMζ in human depression or PTSD, studies on PKMζ in PTSD are ongoing (Coplan and Sacktor), and the results will be of interest in relation to these NHP data.

Last, our results also suggest that the conceptualization of the molecular treatment of PTSD and/or the consequences of early-life adversity is likely more complex than simply the erasure of traumatic memories. Here we see that the VFD paradigm, modeling at least a risk for PTSD (Heim et al. 1997), entails diminution of a molecular representation of memory traces in the ventral hippocampus.

In conclusion, the current report indicates that developmental adversity results in lifelong reductions of PKMζ expression in subregions of the ventral hippocampus in NHPs. We speculate that attenuation of a neural substrate critical to emotional navigation and affective memory maintenance may ensue. An adversity-induced reduction in PKMζ-maintained LTP in the afferent and efferent projections of the ventral hippocampus could, in turn, impair neocortical function in certain areas or circuitries, in line with a caloric "thrift" hypothesis that we have proposed for depression (Coplan et al. 2018). The reduction in the molecular mechanism of LTP maintenance within the ventral hippocampus may therefore be relevant to the underlying mechanisms of mood disorders and posttraumatic disorders.

Materials and Methods

Approvals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2010). The SUNY Downstate Medical Center Institutional Animal Care and Use Committee (IACUC) approved the protocol.

Table 1. Subjects with timid responses to human exposure have lower amounts of protein kinase Mζ (PKMζ) in hippocampus, compared with subjects with confrontational responses

	Timid		Confrontational				
	Mean	SEM	Mean	SEM	$F_{(1,7)}$	P	η_p^2
Moleculare	**15.4**	**1.4**	**22.0**	**1.4**	**9.11**	**0.02**	**0.57**
GC	**15.3**	**0.8**	**19.4**	**0.6**	**9.34**	**0.02**	**0.57**
Hilus	15.7	1.0	18.6	0.8	3.59	0.10	0.34
CA3	**14.5**	**1.1**	**19.1**	**0.7**	**7.89**	**0.03**	**0.53**
CA1	16.4	1.4	20.7	0.5	4.05	0.08	0.37
Subiculum	19.4	1.5	22.1	0.5	1.52	0.26	0.18

Mean ± SEM values of PKMζ immunocytochemical fluorescence intensity are expressed in arbitrary units (Timid, $n = 6$; Confrontational, $n = 3$). Values from subregions with significantly different *P*-values, together with *F*-values and effect sizes are shown in bold.

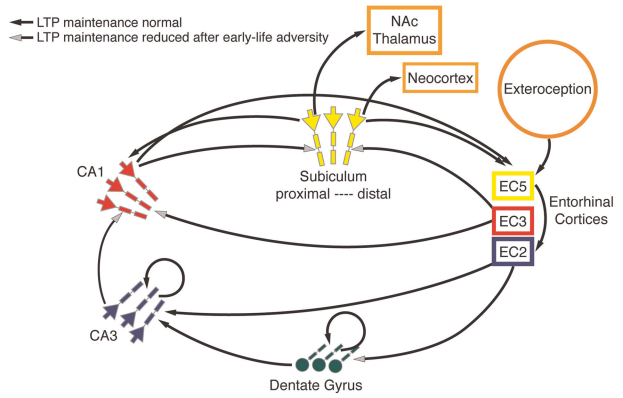

Figure 3. Schematic of the major entorhinal–hippocampal circuit nodes drawn to highlight the nested long and short loops of information flow and processing, with reduced postsynaptic protein kinase Mζ (PKMζ) expression observed after variable-foraging demand (VFD)-induced early-life adversity marked as gray arrowheads. Reduced PKMζ identifies sites of loss of the molecular mechanism of long-term potentiation (LTP) maintenance and experience-dependent information storage, functionally biasing hippocampal processing toward local self-originating activity at the expense of extrinsic entorhinal-originating activity. This VFD-induced bias may promote self-referential information processing resembling rumination. (EC2) Entorhinal cortex layer 2, (EC3) entorhinal cortex layer 3, (EC5) entorhinal cortex layer 5, (NAc) nucleus accumbens.

Subjects

Ten adult males were used to evaluate VFD effects, six of whom had been raised under conditions of VFD and four as controls. The males, placed in single cages in a single rack, were spared any wounding, and subjects of both groups were in good health as per veterinarian assessments. We were not aware of any differences in VFD- versus non-VFD veterinarian assistance.

Variable-foraging demand–rearing procedure

Mother–infant dyads were group housed in pens of five to seven dyads for at least 4 wk prior to the VFD-rearing procedure (Coplan et al. 1996). After infants reached at least 2 mo of age, dyads were subjected to a standard VFD procedure, involving eight alternating 2-wk blocks, in which maternal food was readily obtained (i.e., low-foraging demand), or more difficult to access (high-foraging demand). During high-foraging demand conditions, the mothers had to find food by digging through clean wood chips in a foraging cart, in which food could be accessed by mothers through apertures in the sides of the cart. In the control non-VFD condition, the mothers' food access was continuously low-foraging demand, in which adequate food was always available. There were no differences in weight between either VFD and non-VFD mothers or their infants. However, the unpredictability of foraging conditions prevented VFD mothers from adequately attending to their infants (Coplan et al. 2015). Thus, the effects of the early-life adversity paradigm are thought to occur through the disruption of normative patterns of maternal rearing and infant attachment (Coplan et al. 2006). Following infancy, no further experimental manipulations were performed that could confound the VFD-rearing effects.

Human exposure response

Nine of 10 male macaques examined in the current study were subject to behavioral testing within 6 mo prior to sacrifice and not closer than 1 wk prior to sacrifice. The order of the animals tested was random and not measured. The singly housed animals were briefly exposed to a human intruder, a fear stimulus that is a variation of a previously detailed masked intruder paradigm (Rosenblum et al. 2001). The intruder was the same male experimenter for all subjects, who stood 2 m from the cage, and a female observer, used for inter-rater reliability, stood a meter behind the intruder. Each human exposure lasted 3 min. Emotional responsivity was rated by the two experimenters blind to rearing status using a 3-point scoring scale. To receive a score of "1" for intruder distress, subjects exhibited consistent "confrontational" behaviors including fang-baring, growling, direct eye contact, piloerection, ear-flexing, cage-shaking, and mouth-gaping. A "timid" response, which received a score of "3," was characterized by an animal that was nonconfrontational, averting eye contact, submissive in posture, displaying lip-smacking behaviors, and receding to the back of the cage. Normative confrontational behavior in male macaques is consistent and without intervening timidity. Therefore, we partitioned behavioral responses between those with consistent confrontational behaviors and those with varying degrees of timidity, and subjects with intermediate or alternating levels of confrontational and timid behaviors (score of "2") were classified under "timid." Inter-rater reliability for the scoring system was $\kappa > 0.90$. In general, a timid response, or a posture of subordinance, seeks to avert threat and differs markedly from confrontational responses in which the macaque responds aggressively to threat, mounting a counter-response intended to intimidate the intruder.

Sacrifice

All subjects were sacrificed by transcardiac perfusion with normal saline (500 mL/kg) followed by 4% paraformaldehyde (500 mg/kg) under deep anesthesia with pentobarbital (15 mg/kg, IV). The macaques were born on different dates, and a necropsy team, which is assembled for any terminal study, requires extensive staffing and can only be convened for a limited number of sessions. Therefore, individual life spans will vary. The mean age of the animals at sacrifice was 8.9 yr ± 0.4 yr, and their weight, 10.1 kg ± 0.9 kg; ~9 yr of age represents full adulthood, allowing us to address the question of persistence of PKMζ changes into adulthood.

PKMζ immunohistochemistry

PKMζ immunofluorescence

Left brain sections of ventral hippocampus cut to 40-µm thickness were rinsed three times for 5 min each in phosphate-buffered saline (PBS at pH 7.4), and transferred to PBS with 0.2% Triton (PBS-T) for permeabilization. Sections were incubated for 10 min with 0.1% glycine in PBS to quench free aldehydes, and rinsed again with PBS for 10 min. The sections were then incubated in blocking buffer (5% normal goat serum in PBS) for 1 h and then overnight in primary antibody (1:200, rabbit anti-PKMζ) (Hernandez et al. 2003) in blocking buffer, or blocking buffer alone as control. Sections were rinsed in PBS, four times for 20 min each, and then incubated with goat anti-rabbit Alexa Fluor 488 (1:200) in blocking buffer overnight on a rotator at room temperature in the dark. The sections were rinsed three times in PBS, washed in distilled water, and mounted with DAPI Vectashield Mounting Medium on glass slides with ProLong Gold (Molecular Probes).

Slides were imaged using an Olympus BS52 microscope fitted with the Olympus DP72 camera at 10×. Experimenters were blind to the rearing conditions of the animals. All parameters (pinhole, contrast, and brightness) were held constant for all sections from the same experiment, and the parameters were optimized for each group of control and experimental animals to account for differences in tissue quality. To compare the intensity profile of PKMζ immunostaining between images, we converted the images into grayscale and used the Matlab functions "imread" and "imagesc" to scale the intensity distribution into the full range of a colormap. Background noise was subtracted using averaged region of interest (ROI) measurements from a no primary control slide. Hippocampal complex subregions were identified using a standard rhesus macaque brain atlas (Paxinos et al. 2000) adapted to bonnet macaques. ROIs from each subregion were selected in the grayscale images (Fig. 1B, insert), and mean PKMζ immunointensity calculated using ImageJ analysis software.

PKMζ immunohistochemistry using peroxidase staining

Sections taken from the same hippocampus as immunofluorescence were removed from cyroprotectant, and heat-induced epitope retrieval performed by boiling in citrate buffer (pH 6.0). Sections were washed in 0.2% PBS-T, and endogenous peroxidase activity quenched with 2% H_2O_2. The sections were then blocked in normal serum, incubated in primary PKMζ antiserum (1:500) for 72 h, washed in 0.2% PBS-T, and incubated in secondary antibody (1:200; Biotinylated goat anti-rabbit, Vector Laboratories), in Avidin/Biotin solution (ABC Elite, Vector Laboratories), and chromagen developed using 3,3′-diaminobenzidine (Vector DAB Substrate #SK-4100, Vector Laboratories). The sections were mounted, dehydrated, cleared, and cover-slipped with DPX Mountant (Sigma).

Statistics

The analysis used a general linear model (GLM) approach, using Statistica 13.0. In each statistical comparison, we used treatment group as the categorical variable, and hippocampal region was treated as a repeated measures-dependent variable. We also tested for an interactive effect between test condition X hippocampal region. If the overall GLM was significant, we then proceeded to perform post hoc univariate analysis within the GLM. For the human exposure test condition, responses were dichotomized as "confrontational" or "continually or intermittently timid" (see "Human Exposure Response," above), and used as the categorical variable in a separate GLM and region as a dependent variable. We also tested the interactive effect of the human exposure response by region. Weight and age were not used as covariates because they were not statistically different between test conditions. In the VFD experiment, the mean age was 8.4 yr ± 0.2 yr for VFD group, and 9.6 yr ± 0.8 yr for control group; $t_8 = -1.79$, $P = 0.11$, Cohen's $d = 1.03$. The mean weight was 10.2 kg ± 1.1 kg for VFD group, and 10.5 kg ± 1.8 kg for control group; $t_8 = -0.15$, $P = 0.88$, Cohen's $d = 0.09$. In the human exposure response analysis, the mean age was 9.7 yr ± 1.1 yr for confrontational group, and 8.5 yr ± 0.2 yr for timid group; $t_7 = 1.57$, $P = 0.16$, Cohen's $d = 0.88$. The mean weight was 8.0 kg ± 1.9 kg for confrontational group, and 10.9 kg ± 0.9 kg for timid group; $t_7 = -1.54$, $P = 0.17$, Cohen's $d = 1.0$. Probability of significance was set at $P < 0.05$ (two-tailed).

Competing interest statement

J.D.C. is a speaker for Sunovion, Abbvie, Teva, Otsuka, BMS, and Neurocrine. He has received grants from Pfizer Pharmaceuticals, GSK, Corcept, and Neurocrine. He has served on the advisory board of Otsuka and Lundbeck. No other authors have interests to disclose.

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