Research

PI3-kinase cascade has a differential role in acquisition and extinction of conditioned fear memory in juvenile and adult rats

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The basolateral amygdala (BLA), medial prefrontal cortex (mPFC) circuit, plays a crucial role in acquisition and extinction of fear memory. Extinction of aversive memories is mediated, at least in part, by the phosphoinositide-3 kinase (PI3K)/Akt pathway in adult rats. There is recent interest in the neural mechanisms that mediate fear and extinction in juvenile animals and whether these mechanisms are distinctive from those in adult animals. In the present study, we examined (1) changes in phosphorylation of Akt in the BLA and mPFC after fear conditioning and extinction in juvenile and adult rats and (2) the effect of BLA and mPFC localized inhibition of the PI3K following acquisition and extinction of contextual fear memory. Our results show that Akt phosphorylation is increased following acquisition of contextual fear learning in the BLA but not in the mPFC in adult and juvenile rats. Extinction learning was not associated with changes in Akt phosphorylation. Although there were no differences in the pattern of phosphorylation of Akt either in adult or juvenile rats, microinjection of the PI3K inhibitor, LY294002, into the BLA or mPFC elicited differential effects on fear memory acquisition and extinction, depending on the site and timing of the microinjection, as well as on the age of the animal. These results suggest that PI3K/Akt has a differential role in formation, retrieval, and extinction of contextual fear memory in juvenile and adult animals, and point to developmental differences between adult and juvenile rats in mechanisms of extinction.

Contextual fear conditioning involves association between a novel context (the conditioned stimulus, CS) and an aversive stimulus (the unconditioned stimulus, US), resulting in a robust fear memory, which can be weakened through extinction (Bouton and Nelson 1994; Rescorla 1996; Berman and Dudai 2001; Myers and Davis 2002). Fear memory regulation is mediated by interactions between the basolateral subunit of the amygdala (BLA) and medial prefrontal cortex (mPFC). Recent research regarding the mechanisms underlying extinction across development has pointed to similar involvement of the mPFC in extinction in juvenile and adult animals (Kim and Richardson 2008). Specifically, juvenile rats display adult-like extinction learning by demonstrating renewal and reinstatement (Bouton 2002; Kim and Richardson 2007; Yap and Richardson 2007). However, the mPFC is a late-maturing brain structure, and previous studies have shown that the number of neurons found in the mPFC decreases from adolescence to adulthood (Markham et al. 2007).

We have recently shown that the function of the mPFC in juvenile prepubertal rats is affected differently by stress than in adult animals (Schayek and Maroun 2015). In adult animals stress resulted in impaired extinction and plasticity in the form of long-term potentiation in the mPFC (Maroun and Richter-Levin 2003; Rocher et al. 2004; Markham et al. 2007; Schayek and Maroun 2015), whereas in juvenile animals, a similar stressor was associated with enhanced extinction and enhanced mPFC plasticity (Schayek and Maroun 2015). The BLA, similarly to the mPFC, undergoes changes in juvenile rats (Markham et al. 2007; Rubinow and Juraska 2009). These results may suggest that the interaction between the mPFC and the BLA is different in juvenile and adult animals.

The phosphoinositide 3-kinase (PI3K)/Akt signaling cascade has roles in cell proliferation and the prevention of apoptosis (Datta et al. 1997; del Peso et al. 1997) and evidence suggests that it has an important role in memory formation and plasticity (Kelly and Lynch 2000; Lin et al. 2001, 2003; Sanna et al. 2002; Chen et al. 2005; Karpova et al. 2006).

We have previously reported that aversive memories could be inhibited by PI3K inhibitors. Specifically, the memory of conditioned taste aversion (CTA) was reduced following inhibition of the PI3K/Akt cascade in the insular cortex (Slouzkey et al. 2013). We have also shown (Kritman and Maroun 2012) PI3K/Akt inhibition in the BLA differentially affects fear acquisition and extinction depending on inhibitor injection time. Whereas the inhibition of PI3K/Akt after acquisition of fear conditioning enhanced the formation of fear memory, its inhibition before the retrieval of the fear memory was associated with reduced fear. In contrast, inhibition of PI3K/Akt after a retrieval session in the infra-limbic subregion of the mPFC (IL-mPFC) was associated with impairments in extinction.

In the present study, we focus on the role of the PI3K/Akt cascade in the BLA and mPFC of juvenile rats and compare the effects to temporally equivalent microinjections of a PI3K/Akt inhibitor into the adult animal. To that end, we examined (1) the changes in phosphorylation of Akt after fear conditioning and extinction in the BLA and mPFC and (2) whether PI3K/Akt inhibition in the BLA or the IL-mPFC differentially affects the formation of fear memory and its extinction.

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Results

Experiment 1: changes in Akt phosphorylation in the BLA and mPFC of adult and young animals following fear and extinction

Samples of BLA and mPFC were taken from the same animal either following fear acquisition or extinction of fear to monitor changes in Akt phosphorylation. As we were interested to compare whether differences exist between juvenile and adult animals, analysis was conducted either on the BLA (Experiment 1A) or on the mPFC (Experiment 1B) following fear and extinction.

Experiment 1A: acquisition of fear memory is associated with an increase in Akt phosphorylation in the BLA in adult and juvenile rats

Animals of both age groups were allocated to one of three protocol groups: naïve (Na; Na adult $n = 13$, Na Juvenile $n = 16$), fear conditioning (FC; FC adult $n = 12$, FC Juveniles $n = 16$), and extinction (EX1; EX1 adult $n = 13$, EX1 Juveniles $n = 11$). Both groups of FC and EX1 underwent fear conditioning, and the EX1 group additionally underwent one extinction session (Maroun et al. 2012). The FC and EX1 groups were decapitated 15 min following fear conditioning and extinction sessions, respectively. Naive animals, who were not behaviorally tested or handled, were decapitated at the same time point as the EX1 group (Figs. 1, 2A).

Akt phosphorylation in BLA samples extracted from rats decapitated following fear conditioning or following subsequent extinction were analyzed by western blot analysis (normalized naive rats of each age group). Two-way ANOVA (Age and Behavioral Protocol) showed a significant effect of protocol ($F_{(2,81)} = 3.4$; $P < 0.05$; Fig. 2B), but no significant differences in age ($F_{(1,81)} = 1.8$; ns) or age × protocol interaction ($F_{(2,81)} = 0.5$; ns). These results indicate that Akt phosphorylation changes as a function of behavioral protocol but not age. Post hoc analysis showed that the FC group was significantly different from all other groups, with increased Akt phosphorylation levels ($P < 0.05$). Total Akt protein levels (normalized to actin) were not affected by protocol ($F_{(2,81)} = 0.13$; ns), age ($F_{(1,81)} = 0.34$; ns), or interaction between age and protocol ($F_{(2,81)} = 0.16$; ns) (see Fig. 2C).

To verify similar levels of Akt phosphorylation in naive animals from the two age groups, independent *t*-test was performed. This analysis showed no significant differences ($t_{27} = -0.83$; NS; naive adult = 1 ± 0.04, naive Juveniles = 1.01 ± 0.08].

Experiment 1B: acquisition and extinction of fear memory do not affect Akt phosphorylation in the mPFC in adult and juvenile rats

Animals of both age groups were allocated to one of three protocol groups: Na (Na adult $n = 9$, Na Juveniles $n = 9$), FC (FC adult $n = 11$, FC Juvenile $n = 10$), and EX1 (EX1 adult $n = 10$, EX1 Juveniles $n = 10$). The level of Akt phosphorylation in samples of mPFC obtained after the different behavioral manipulations was determined by western blot with normalization to naive rats from the same age group (Fig. 3A). Two-way ANOVA showed no significant effect of age ($F_{(1,59)} = 0.2$; ns), behavioral protocol ($F_{(2,59)} = 0.3$; ns; Fig. 3B), or interaction between age × protocol ($F_{(2,59)} = 0.06$; ns), indicating that phosphorylation of Akt does not change as a function of behavioral protocol or age.

The total amount of Akt protein (normalized to actin; see Fig. 3C) was not affected by protocol ($F_{(2,81)} = 0.11$; ns), age ($F_{(1,81)} = 0.26$; ns), or the interaction between age and protocol ($F_{(2,81)} = 0.07$; ns); when the results were normalized to those of naive rats of each age group. Similarly, no significant differences between the naive groups were found ($t_{16} = -0.98$; NS; naive adult = 1 ± 0.1 naive juveniles = 1.18 ± 0.15).

Experiment 2: inhibition of PI3K/Akt in the BLA after fear conditioning results in differential effects on acquisition and extinction in adult and juvenile animals

We have previously reported (Kritman and Maroun 2012) that inhibition of PI3K in the BLA of adult animals after fear conditioning is associated with increased freezing and impaired extinction of contextual fear memory. In this experiment, we aimed to replicate this finding and to compare the effect in adult and juvenile rats. For this purpose, rats underwent contextual fear conditioning, and 15 min after the end of the session had PI3K inhibitor LY294002 (LY) or vehicle (VH) microinjected into the BLA (adult VH $n = 7$, adult LY $n = 8$; juveniles VH $n = 8$, juveniles LY $n = 12$). For the subsequent 3 d, animals underwent 10 min extinction sessions, and freezing levels were measured (Fig. 4A).

Two-way ANOVA with repeated measures showed significant effects of treatment ($F_{(1,31)} = 9.274$; $P < 0.01$), interaction between Group × age ($F_{(1,31)} = 7.9$; $P < 0.01$) (Fig. 4B), but no significant effect of age ($F_{(1,31)} = 0.3$; ns). In addition, there was a significant effect of Testing day ($F_{(2,62)} = 40.3$; $P < 0.001$), Testing day × Treatment ($F_{(2,62)} = 12.1$; $P < 0.001$), and Testing day × age interactions ($F_{(2,62)} = 9.4$; $P < 0.001$). There was no significant effect of the interaction between Testing days × age × Treatment ($F_{(2,62)} = 0.6$; ns).

To understand the source of the significant interaction between age and Treatment, and since we were interested in the way inhibition of PI3K modulates fear memory and extinction within each age group, we analyzed the data of each age group separately. ANOVA with repeated measures indicated that in adult rats there were significant effects of Treatment ($F_{(1,13)} = 10.5$; $P < 0.01$), and Testing day ($F_{(2,26)} = 27.1$; $P < 0.001$), but no significant interaction between Treatment × Testing day ($P > 0.05$). Specifically, the LY-treated group showed

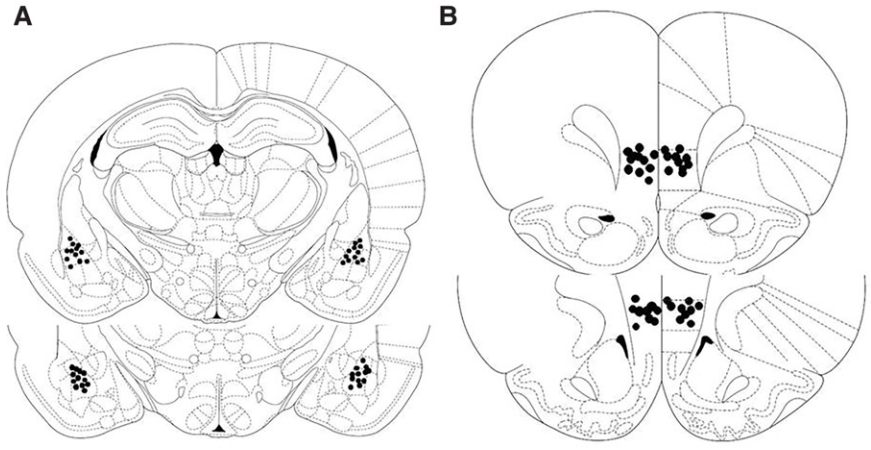

Figure 1. Position of cannulae and injection sites. Solid black circles indicate location of cannulae in (*A*) the basolateral amygdala (BLA, coronal view at positions 3.14 and 3.30 mm posterior to bregma) and (*B*) the infralimbic subregion of the mPFC (IL-mPFC, coronal view at positions 3.20 and 2.70 mm anterior to bregma).

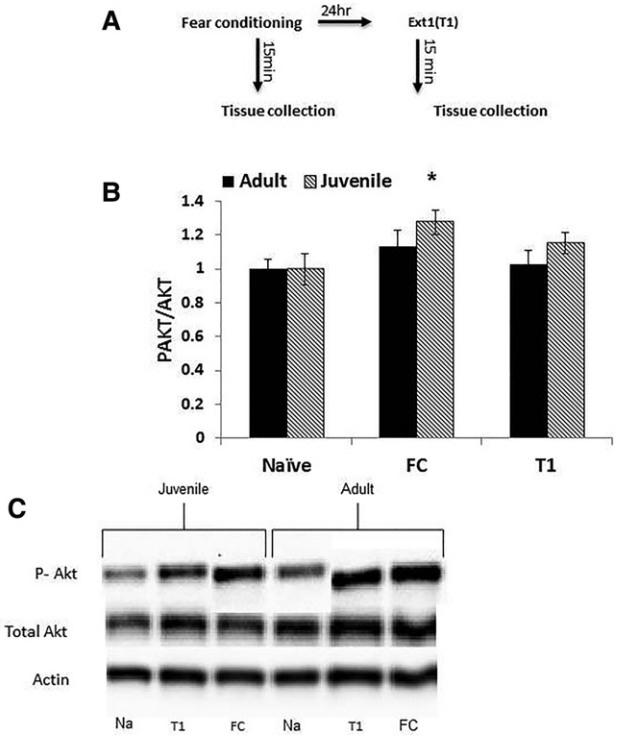

Figure 2. Contextual fear conditioning is associated with an increase in Akt phosphorylation levels in the BLA in adult and juvenile rats. (*A*) Schematic representation of the experimental protocol. (*B*) Akt phosphorylation levels in relation to total Akt levels normalized to the naive group, presented as mean ± SEM. The results point to an increase in the phosphorylation of Akt in the FC group compared with the naive group ($P < 0.05$). No significant changes in phosphorylation levels of Akt after the first extinction day were detected. (C) Representative blots of total Akt, P-Akt, and actin (naive [Na], after fear conditioning [FC]; after the first non-reinforced tests [T1]). (*), different from naive.

increased freezing levels on the three testing days (T1 = 94.4% ± 1.8%, T2 = 83.4% ± 8%, T3 = 59.4% ± 11.9%) when compared with the VH-treated group (T1 = 80% ± 7.6%, T2 = 40.1% ± 9.5%, T3 = 27.4% ± 6.1%). The results show that inhibition of PI3K/Akt in the BLA after acquisition of contextual fear memory in the adult animal enhances freezing levels during the testing days in comparison to the control group.

ANOVA with repeated measures of the juvenile rats showed no significant effect of Treatment ($F_{(1,18)} = 0.5$; ns), but significant effect of Testing day ($F_{(2,36)} = 10.4$; $P < 0.001$) and a significant Treatment × Testing day interaction ($F_{(2,36)} = 13.1$; $P < 0.001$). Inspection of the results showed that microinjection of LY294002 into the BLA was associated with significantly reduced freezing levels only at the T1 session (VH = 78.2% ± 4.9%, LY 57.6% ± 4.5%: $P < 0.05$), but not at T2 (VH 57.6% ± 5.3%, LY T2 = 70.1% ± 4.6%) or T3 (VH T3 = 45.8% ± 5.7%, LY T3 = 57.6% ± 4.5%). The results suggest that inhibition of PI3K in the BLA after acquisition of contextual fear memory transiently impairs retrieval of fear memory in juvenile rats.

Experiment 3: PI3K/Akt inhibition in the BLA before the retrieval of fear memory reduces freezing levels in adult rats but enhances freezing levels in juvenile rats

It was previously shown that inhibition of PI3K in the hippocampus or the BLA is associated with impaired retrieval of fear memory (Chen et al. 2005; Kritman and Maroun 2012). The results of Experiment 2 showed that the adult and juvenile rats responded differently to inhibition of PI3K/Akt in the BLA after acquisition of fear memory. Here, we wanted to examine how inhibition of PI3K/Akt in the BLA before retrieval of fear memory would affect retrieval and subsequent extinction sessions in juvenile animals as compared with the adults (Fig. 5A).

Rats of both age groups underwent contextual fear conditioning and 24 h later and 15 min prior to the first retrieval session, they had the PI3K/Akt inhibitor LY294002 (LY) or vehicle microinjected into the BLA (adult VH $n = 13$, adult LY $n = 16$; juvenile VH $n = 9$; juvenile LY $n = 10$). A further 24 and 48 h later, animals were subjected to additional 10-min extinction sessions.

Two-way ANOVA with repeated measures showed significant effects of age ($F_{(1,44)} = 15.7$; $P < 0.001$) and the interaction between Treatment × age ($F_{(1,44)} = 17.6$; $P < 0.001$; Fig. 5B), but no significant effect of Treatment (ns).

To understand the source of the significant interaction between age and Treatment, we analyzed the data for each age separately. ANOVA with repeated measures indicated that in adult rats there were significant effects of Treatment ($F_{(1,27)} = 9.5$; $P < 0.01$) and Testing day ($F_{(2,54)} = 19$; $P < 0.001$), but no significant interaction of Treatment × Testing day ($P > 0.05$), suggesting that the groups extinguished the fear conditioning memory similarly across the testing days. The LY-injected group showed reduced freezing levels compared to the vehicle-treated group (LY: T1 = 54.2% ± 6.2%, T2 = 31.9% ± 6%, T3 = 35% ± 4.7%; VH: T1 = 82.2% ± 3.5%, T2 = 57% ± 8.4%, T3 = 43.6% ± 7.7%). These results suggest that inhibition of PI3K/Akt in the BLA of

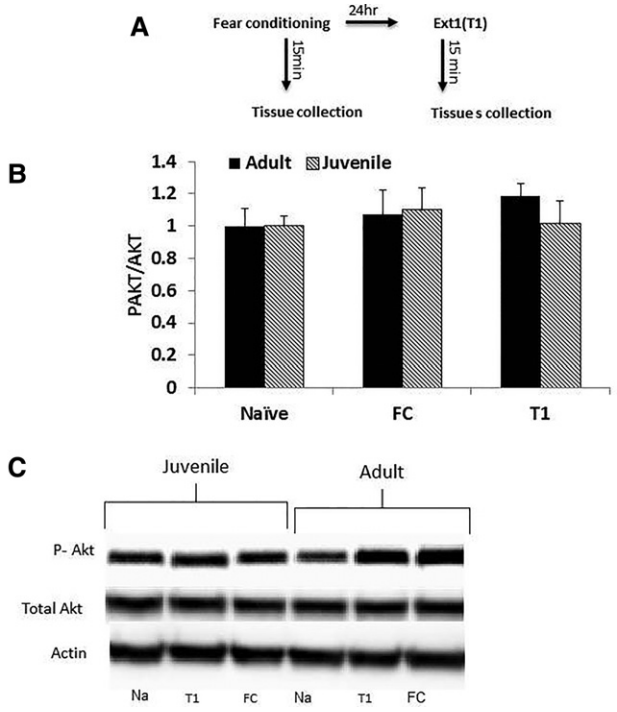

Figure 3. Akt phosphorylation does not change in the mPFC following fear conditioning and extinction. (*A*) Schematic representation of the experimental protocol. (*B*) Akt phosphorylation levels in relation to total Akt levels normalized to the naive group, presented as mean ± SEM. The results show that fear conditioning and extinction in both groups of age were not associated with changes in phosphorylation of Akt compared with the relevant naive group. (*C*) Representative blots of total Akt, P-Akt, and actin (naive [Na], after fear conditioning [FC]; after the first non-reinforced tests [T1]).

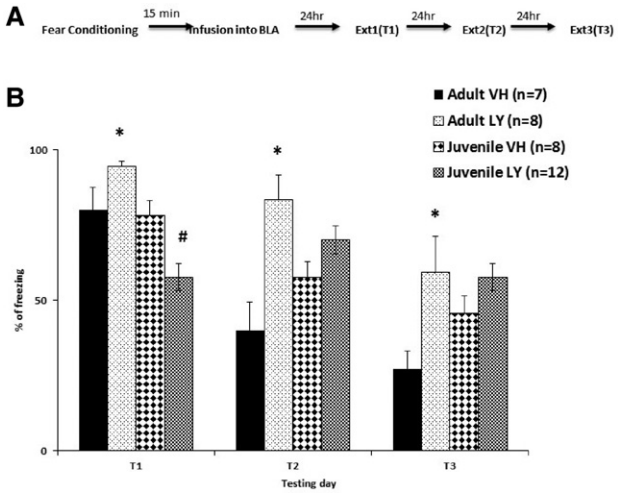

Figure 4. Inhibition of PI3K/Akt in the BLA after fear conditioning results in differential effect on acquisition and extinction in adult and juvenile animals. (*A*) Schematic representation of the experimental protocol. (*B*) The PI3K inhibitor LY294002 was microinjected into the BLA 15 min after fear conditioning in both age groups. On three consecutive days the groups underwent 10-min sessions of extinction. There was a significant interaction between age and the treatment ($P<0.01$). In the adult rat, PI3K/Akt inhibition resulted in an increase in freezing levels on all 3 d compared with vehicle. In the juvenile rats, there was a transient decrease in freezing level only on the retrieval test day in the LY injected group compared with vehicle and no differences on the next extinction days. Results are represented as mean ± SEM. (*), different from VH adult. (#), different from VH juvenile.

adult animals before retrieval of contextual fear accelerates the reduction in freezing levels.

In the juvenile animals, ANOVA with repeated measures showed significant effects of Treatment ($F_{(1,16)} = 8.8$; $P<0.01$) and Testing day ($F_{(2,32)} = 15.5$; $P<0.001$), but no significant Treatment × Testing day interaction ($P>0.05$). The results show that the LY-injected group have increased freezing levels compared with the vehicle-treated animals (LY: T1 = 96.6% ± 2.2%, T2 = 79.7% ± 6%, T3 = 70.3% ± 7.6%; VH: T1 = 76.1% ± 4.9%, T2 = 61% ± 7.7%, T3 = 42.4% ± 4.9%). These results suggest that inhibition of PI3K/Akt in the BLA before retrieval of contextual fear enhances freezing levels.

Experiment 4: PI3K/Akt inhibition in the IL-mPFC before the retrieval of fear memory has no effect on freezing behavior

Interactions between the BLA and mPFC subserve fear memory regulation (for review, see Maroun 2013). In the next experiments, we aimed to examine how inhibition of PI3K/Akt in the mPFC would affect extinction memory. Rats of both age groups underwent contextual fear conditioning, and 24 h later, 15 min prior to the first testing session, the PI3K/Akt inhibitor LY294002 (LY) or vehicle was microinjected into the IL-mPFC (adult VH $n=8$, adult LY $n=9$; juvenile VH $n=8$, juvenile LY $n=12$). The animals underwent additional 10 min extinction sessions 24 and 48 h later (Fig. 5A).

Two-way ANOVA with repeated measures showed a significant effect of age ($F_{(2,66)} = 5.4$; $P<0.001$) but no significant effect of Treatment or Treatment × age interaction ($P>0.05$; Fig. 5C]. In contrast, there were significant effects of Testing day ($F_{(2,66)} = 65$; $P<0.001$) and Testing day × age ($F_{(2,66)} = 5.1$; $P<0.01$), but no significant effect of Testing day × Treatment or Testing day × age × Treatment interaction (ns). Analysis of the significant interaction between Testing day and age showed significant differences only at T3 with the freezing levels of the juveniles being significantly higher than those of the adults (Juvenile: 58.1% ± 3.6%; Adults: 33.3% ± 6.0%; $P<0.01$).

These results suggest that inhibition of PI3K/Akt in the IL-mPFC before retrieval of contextual fear has no effect on adult or juvenile rats, and further show that the kinetics of extinction may be different in both ages.

Experiment 5: PI3K/Akt inhibition in the BLA after the first non-reinforced retrieval test is associated with impaired extinction in juvenile rats

To examine the role of the PI3K/Akt cascade in extinction consolidation, we targeted the consolidation window by microinfusion of the inhibitor after the first testing session. Rats of both age groups were subjected to contextual fear conditioning and 24 h later underwent the first non-reinforced test. Fifteen minutes after

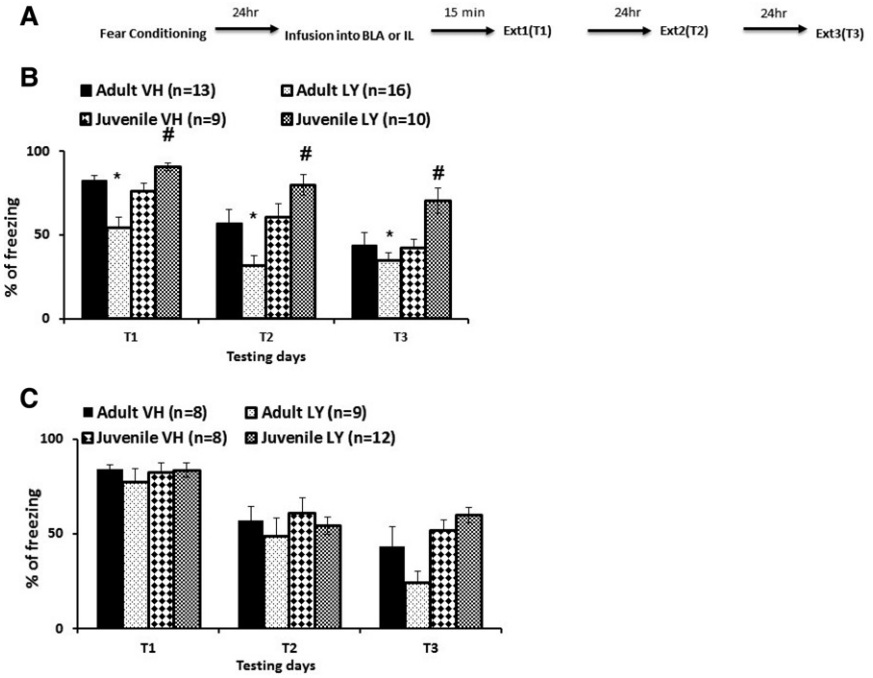

Figure 5. PI3K/Akt inhibition before the retrieval of fear memory reduces freezing levels in the adult rat as opposed to enhanced freezing levels in the juvenile rat only when micro infused in the BLA. (*A*) Schematic representation of the experimental protocol. (*B*) Animals of both age groups were microinjected with either LY294002 or vehicle into the BLA 15 min before the retrieval of fear memory on T1. There was a significant difference between the age groups and interaction between Treatment × age. The adult group showed decrease in freezing levels on all 3 d versus increased freezing levels in juvenile rats in all 3 d. (*), different from VH adult. (#), different from VH juvenile. (*C*) LY294002 or vehicle was microinjected into the IL-mPFC 15 min before the retrieval of fear memory on T1. There was no significant difference between the groups. Results are represented as mean ± SEM.

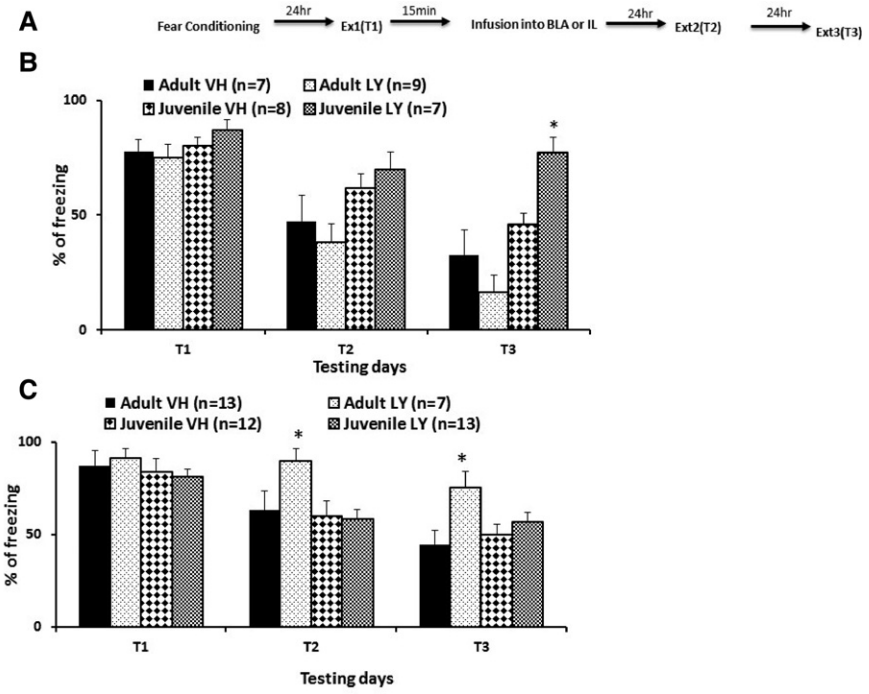

Figure 6. PI3K/Akt inhibition in the BLA after the first non-reinforced retrieval impairs extinction in juvenile rats, whereas in the IL it impairs extinction in adult animals. (*A*) Schematic representation of the experimental protocol. (*B*) Animals were microinjected into the BLA with PI3K/Akt inhibitor LY2940020 or vehicle 15 min after T1. There was no significant difference between the groups on T1. In contrast, there was a significant interaction between Treatment × age. Whereas in adult animals, there was no effect of drug treatment, LY-treated juvenile rats exhibited increased freezing levels on T3, but not on T2. Results are represented as mean ± SEM. (*), different from VH juvenile. (*C*) Animals were microinjected into the mPFC with PI3K/Akt inhibitor LY2940020 or vehicle 15 min after T1. There was significant interaction between Treatment and age. Only in adults, LY-treated animals showed enhanced freezing levels. Results are represented as mean ± SEM. (*), different from VH-adult.

the end of the test, animals were microinjected with LY294002 or the relevant vehicle into the BLA (adult VH $n = 7$, adult LY $n = 9$; juvenile VH $n = 8$, juvenile LY $n = 7$). The animals underwent two additional 10-min extinction sessions after 24 and 48 h (Fig. 6A).

Factorial ANOVA of freezing levels during the first extinction test (retrieval of the fear memory) (adult [VH = 77.7% ± 5.4%, LY = 75.2% ± 6.4%]; Juvenile [VH = 80.2% ± 3.3%, LY = 87.1% ± 4.4%]) showed no significant main effect for any of the variables or for the interaction between them (Treatment: $F_{(1,27)} = 0.2$, ns; age: $F_{(1,27)} = 2.3$, ns; age × Treatment $F_{(1,27)} = 0.99$, ns), which suggests comparable freezing levels during the retrieval session.

Two-way ANOVA with repeated measures at 24 h (T1) and 48 h (T2) following the microinjection revealed significant effects of age ($F_{(1,27)} = 19$; $P < 0.001$) and Treatment × age interaction ($F_{(1,27)} = 5.6$; $P < 0.05$) (Fig. 6B), but no significant effect of Treatment (ns).

Further investigation of the results for each age using ANOVA with repeated measures on T2 and T3 did not show significant effects of Treatment or interaction ($P > 0.05$) in the adult animals, but showed significant effect of Testing day ($F_{(1,14)} = 5.4$; $P < 0.05$). These results show that the adult LY-injected group was not different compared with the VH-injected group, and that both groups extinguished the fear conditioning memory similarly across the testing days.

In juvenile rats, the results showed significant effects of Treatment ($F_{(1,13)} = 5.3$; $P < 0.05$) and Treatment × Testing day interaction ($F_{(1,13)} = 15.1$; $P < 0.005$), but no significant effect of Testing day ($F_{(1,13)} = 1.9$; ns). An independent *t*-test showed that administration of LY294002 into the BLA was not associated with differences at T2 (VH = 61.6% ± 6.6%; LY = 69.8% ± 7.5%), but with enhanced freezing levels at T3 (VH = 45.8% ± 5.2%; LY = 77.3% ± 6.5%, $P < 0.05$). These results suggest that inhibition of PI3K/Akt in the BLA after acquisition of extinction of contextual fear memory impairs extinction learning in juvenile rats in a delayed manner.

Experiment 6: PI3K/Akt inhibition after the retrieval of fear memory in the IL-mPFC enhanced freezing levels in adult animals only

Rats of both age groups (adults VH $n = 13$, adults LY $n = 7$, juvenile VH $n = 12$, juvenile LY $n = 13$) were subjected to contextual fear conditioning and 24 h later underwent the first retrieval session. Fifteen minutes after the end of the session LY294002 or the relevant vehicle was microinjected into the IL-mPFC. Animals underwent two additional 10-min extinction sessions after a further 24 and 48 h (Fig. 6A).

Factorial ANOVA of freezing levels during the first extinction test (retrieval of the fear memory) showed no significant main effect of any of the variables or of the interaction between them (Treatment: $F_{(1,41)} = 1.4$, ns; age: $F_{(1,41)} = 0.3$, ns; age × Treatment $F_{(1,\ 41)} = 0.2$, ns), suggesting comparable freezing levels during the retrieval of fear memory.

Two-way ANOVA with repeated measures on the two consecutive days following the microinjection revealed significant differences of Treatment ($F_{(1,41)} = 7.0$; $P < 0.05$) and significant interaction ($F_{(1,41)} = 4.3$; $P < 0.05$), but no significant effect of age ($P > 0.05$) (Fig. 6C).

To better understand the interaction, results were analyzed by the age factor. ANOVA with repeated measures showed that only in the adult group the vehicle- and LY-treated group differed ($F_{(1,18)} = 5.6$; $P < 0.001$), while there was no significant difference in the juvenile group ($F_{(1,23)} = 0.2$; ns). Specifically, the LY-adult group showed impaired extinction when compared with the vehicle-treated group (VH: 53.78% ± 5.3%; LY: 80.47% ± 7.29% average for the two extinction days) (Fig. 6C).

These results suggest that inhibition of PI3K/Akt in the IL-mPFC impairs extinction consolidation in adult animals but not in juvenile animals.

Discussion

The PI3K/Akt pathway, a downstream target of neurotrophin, has been traditionally suggested as a major mediator of cell growth, survival, and metabolism in neurons (Kandel and Hay 1999; Brunet et al. 2001). Emerging evidence points to a critical role of the PI3K/Akt pathway in the formation of memory (Lin et al. 2001; Chen et al. 2005; Slouzkey et al. 2013). The results presented in this study confirm the role of PI3K/Akt in fear and extinction memory, and show complex involvement of this pathway in these

processes in adult and juvenile animals. Similarities and differences in the role of PI3K/Akt in the two age groups will be discussed in the next sections.

Changes of pAKT following fear and extinction

The biochemical findings of the present work show that Akt phosphorylation in the BLA changes as a function of behavioral protocol and not age. Namely, animals from both age groups that underwent fear conditioning exhibited increased levels of Akt phosphorylation compared with naive rats. These results are similar to the result shown by Lin et al. (2001), who showed increased phosphorylation of Akt in the BLA after a potentiated startle paradigm, and are consistent with our previous work using the CTA paradigm, showing an increase in Akt phosphorylation levels in the insular cortex following acquisition of CTA (Slouzkey et al. 2013). These results also confirm the involvement of the BLA in the formation of the fear memory in young animals (Herry et al. 2010) and suggest that the molecular machinery, at least when testing PI3K, is similar in adults and juveniles (Lobo et al. 2006).

In the mPFC, Akt phosphorylation levels did not change as a function of behavioral protocol or age, indicating that the change in Akt phosphorylation after the formation of fear memory in both age groups is restricted to the BLA. This may reinforce the notion that the mPFC is not involved in the formation of fear memory (e.g., Milad and Quirk 2002).

Extinction training, in contrast to fear formation, was not associated with any detectable change in Akt phosphorylation. That extinction does not affect Akt phosphorylation in either the BLA or the mPFC is in contrast to studies carried out by ourselves and others that showed that extinction of either CTA or potentiated startle response is associated with reduced Akt phosphorylation in the insular cortex (Slouzkey et al. 2013) or the BLA (Lin et al. 2003), respectively. It should be noted however that Lin et al. (2003), who used a potentiated startle paradigm (light plus noise), reported that conditioning resulted in increased Akt phosphorylation levels 60 min after the end of the acquisition session; however, 10 min after the presentation of light alone (extinction session), there was a decrease in the Akt phosphorylation before a return to baseline levels, comparable with the unpaired group (Lin et al. 2003). Future studies should attempt to use similar behavioral protocols to examine whether extinction is indeed associated with changes in Akt phosphorylation since, for example, differences between contextual fear conditioning, potentiated startle response, and CTA could be due to the fact that each of these paradigms recruits its own constellation of neuronal, cellular, and molecular bases (for review, see Davis and Myers 2002; Myers and Davis 2002).

We also show here that there are no differences between the two age groups either in the baseline levels of Akt phosphorylation or in the pattern of changes after fear conditioning and extinction. A previous study addressing the magnitude of contextual fear memory in mice at different postnatal range of ages reported that 29 PND mice do not show contextual fear memory (Pattwell et al. 2011). Interestingly, these 29 PND mice showed no changes in phosphorylated Akt (pAkt) expression in the hippocampus following the retrieval of fear while adults had increased pAkt expression following fear memory retrieval. This further confirms the idea that changes in pAkt levels are indicative of fear memory formation, however, contrasts our findings that PND30 rats are able to form fear memory and that this is associated with changes in the pAkt levels in the BLA. It should be noted that Akers et al. (2012) who studied the ontogeny of contextual fear memory contradicted the findings of Pattwell et al. (2011) by showing that the formation of memory was stable also in adolescent mice (see also review of Hefner and Holmes 2007).

Differences in the role of PI3K in adult and juvenile animals

Although the pattern of changes in Akt phosphorylation is similar in both adult and juvenile rats, the major finding of this study was that inhibition of PI3K/Akt elicited differential effects on fear conditioning and extinction, depending on the brain area, time of inhibitor microinjection and animal age. In adult animals, microinjection of LY294002 into the BLA after acquisition of conditioning enhanced the formation of fear memory as demonstrated by higher freezing levels in the inhibitor-treated animals when compared with the vehicle-treated group, consistent with our previous findings (Kritman and Maroun 2012). Similar inconsistency in the adults group between the biochemical and the pharmacological data was also reported in our previous work (Slouzkey et al. 2013). This inconsistency between the biochemical and pharmacology in both our studies could suggest that PI3K is involved, but not obligatory, in the formation of memory. The PI3K/Akt cascade has been shown to interact with the MAPK/extracellular signal-regulated kinase (ERK) pathway under certain conditions (Duckworth and Cantley 1997; Perkinton et al. 1999). It was previously shown that long-term potentiation (LTP), the hypothesized cellular model for memory formation, in the dentate gyrus is associated with PI3K activation and that blockage of PI3K/Akt blocks LTP maintenance (Horwood et al. 2006). The PI3K/Akt pathway is independent of the MAPK/ERK pathway as ERK1/2 is normally hyperphosphorylated. Possibly, when the PI3K/Akt pathway is inhibited the formation and extinction of fear memory may be mediated by the MAPK/ERK pathway (Schafe et al. 2000; Herry et al. 2006).

It should be noted that in juvenile animals, the inhibition of PI3K after fear conditioning resulted impaired retrieval of fear, hinting that the formation of fear is dependent on PI3K activation; however, this effect was transient as the next day there was no differences between the two groups.

The amygdala has an established role in extinction in juvenile rats since it has been shown that temporary inactivation of the amygdala, by injection of bupivacaine, results in impaired extinction retention in juvenile rats (Kim and Richardson 2008). To further examine the role of the amygdala in retrieval and extinction of fear memory in juvenile rats, we compared the effect of microinjection of the inhibitor LY294002 into the BLA before and after the first retrieval test in adult and juvenile rats. In adults, PI3K/Akt inhibition before retrieval resulted in reduced freezing levels, suggesting inhibition of fear memory as we previously reported (Kritman and Maroun 2012). In the juvenile rats, the same PI3K/Akt inhibition before retrieval resulted in increased freezing that persisted over the testing days, demonstrating enhanced freezing levels and differences compared with the control group. Inhibition of PI3K/Akt in the BLA after the retrieval test, which aimed to target the consolidation phase, impaired extinction in the juvenile rats, whereas in the adult rats no effect on consolidation of extinction was observed. These results suggest opposing effects between the two age groups, consistent with previous findings (for review, see Spear 2000). It was previously shown that adolescents differ from adults in their response to a variety of drugs. As indicated in Spear (2000), these ontogenetic variations in drug responsivity may be related in part to age-associated differences in pharmacokinetics. Changes in body composition and organ function associated with the adolescent growth spurt and rising gonadal steroid titers may alter the volume of drug distribution and drug metabolism and excretion rates (Hein 1987). Similarly, Tsai et al. (2014) reported that the same stress paradigm applied to adult or juvenile rats differentially affected their amygdala-dependent memory tasks, local neuron morphology, and protein expression of brain-derived neurotrophic factor (BDNF)-TrkB. Taken together, the results suggest that

PI3K inhibition at the BLA level has opposing effects in the two age groups.

In the present study, inhibition of PI3K/Akt into the IL before the first extinction session resulted in no differences between the naive and treated groups in either adult or juvenile rats; however, inhibition after T1 resulted in impaired extinction only in the adults. In juvenile animals, there were no significant differences between the two groups. These findings suggest that (1) PI3K/Akt in the IL is not engaged in retrieval of fear memory, (2) IL is not involved in this process either in adult or in juvenile rats, and (3) in the adult animal, IL plays a role in the consolidation of extinction and PI3K has a functional role in the consolidation. These findings are consistent with previous reports showing that the IL-mPFC plays a role in the consolidation of extinction rather than in the acquisition phase and confirms the effects of inactivation or pharmacological blockades (e.g., Santini et al. 2001, 2004). The lack of effect in juvenile animals may suggest that the PI3K pathway in the IL is not involved in extinction consolidation at this age. Interestingly, in adult animals, the induction of LTP in the mPFC by the application of high-frequency stimulation (HFS) results in an increase in Akt phosphorylation (Sui et al. 2008) and changes in the magnitude of LTP in the mPFC correlated to more efficient extinction (Cho et al. 2013). In juvenile animals, however, we previously reported that the application of HFS induced very moderate levels of LTP in the juvenile animal, suggesting differences in the magnitude of LTP between adults and juveniles (Scahyek and Maroun, 2015). Together, these results could suggest that plasticity and changes in the Akt phosphorylation in the mPFC that underlie extinction are age-dependent. In confirmation of this hypothesis, adolescent animals (35PND) that showed poor extinction retention had no activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway in the mPFC (Kim et al. 2011). This could suggest that the mPFC was not activated under the specific training/extinction procedures used (McCallum et al. 2010) and that stronger stimuli should be applied for the activation of the mPFC. Although the kinetics of extinction in juvenile animals are intact in the present study, it should be noted that juvenile animals showed a tendency for lower extinction rates compared with the adults, and this is in accordance with previous research that showed that in place preference paradigms adolescent rats require more time than adults to extinguish conditioned associations (Brenhouse and Andersen 2008).

Our results show differential involvement of the PI3K/Akt pathway in the BLA and mPFC of adult and juvenile animals. Some mirror effects between the two age groups were found that hint to a switch in the role of each of the IL cortex and BLA.

Together, the findings of the present study provide further evidence to our claim that the circuit mediating fear and extinction is distinctive in juvenile pups compared with adult animals, or at least, reacts differently in the two age groups. From a clinical perspective, our results suggest that caution should be taken when considering treatments of young children when compared with adults in stress-related disorders.

Materials and methods

Animals

Male Sprague Dawley rats were bred and housed at the University of Haifa. Rats were housed with their littermates and mother. At the age of 21 d, they were weaned and housed separately from their mother in Plexiglas cages and maintained on a free-feeding regimen. The cages were kept at 22 ± 2°C under 12/12 h light/dark cycles, and all tests were performed between 10:00 and 16:00 hours. The juvenile rats had cannulae implanted at an age of 24 d and were fear conditioned at an age of 30 d (weight at time of experiment 75–100 g). Adult rats had cannulae implanted when 53 d old and 60 d old at the time of behavioral testing (300–400 g). The experiments were approved by the University of Haifa Ethics and Animal Care Committee, and adequate measures were taken to minimize pain or discomfort, in accordance with the guidelines laid down by the US NIH regarding the care and use of animals for experimental procedures. All rats underwent a handling-familiarization session before the beginning of the experiment.

Surgery

Adult (53 d of age) and juvenile rats (24 d) were anesthetized using ketamine and xylazine (65 and 7.5 mg per kg i.p., respectively). For the juvenile rats, ketamine and zylazine were diluted 1:10 with saline before the injection and then were injected in the same proportion as the adult. The rats were restrained in a stereotaxic apparatus (Stoelting) and implanted bilaterally with stainless steel guide cannulae (23 gauge, thin wall) aimed at the following coordinates. In the adult rats, cannulae were implanted bilaterally into the BLA (anteroposterior, −3.0 mm relative to bregma; lateral ± 5.0 mm; ventral, −7.6 mm) (Paxinos and Watson 1998) or the IL-mPFC (anteroposterior, +3.2 mm; lateral, ±0.5 mm; ventral, −4.6 mm). In juvenile rats, cannulae were implanted bilaterally into the BLA (anteroposterior, −2.6 mm relative to bregma; lateral, ±4.8 mm; ventral, −6.0 mm) (Paxinos and Watson 1998) or the IL-mPFC (anteroposterior, +2.7 mm relative to bregma; lateral, ±0.6 mm; ventral, −3.8 mm). The cannulae were held in place with acrylic dental cement and secured with two skull screws. A stylus was placed in the guide cannula to prevent clogging. After surgery rats were subcutaneously injected with an antibiotic (Pen-strep 20/25 veterinary, 0.05 mL) and a painkiller (Calmagin at 0.02 mL per 100 g). Whereas the adult animals were allowed 1 wk to recuperate before being subjected to experimental manipulations, the juvenile animals were given 6 d to recuperate.

The locations of cannulae in the BLA and the mPFC were verified histologically (Fig. 1) and only animals in which cannulae were correctly placed in the BLA or the mPFC were included, three juvenile animals had misplaced cannulae in the mPFC, and five in the BLA and two adult animals had their cannulae misplaced in the BLA.

Contextual fear conditioning and extinction

Contextual fear conditioning was carried out using the Startle Fear system (Panlab Spain), in which each conditioning chamber has black plastic walls and a metal grid floor. Rats were placed in the conditioning chamber (conditioned stimulus) and, after 2 min exploration time, were given three foot shocks of 0.4 or 0.6 mA for juvenile or adult rats, respectively, with 2 min separation intervals. After each conditioning session, the shock grids and the walls were cleaned with 70% ethanol and dried with a paper towel. Unless otherwise indicated, animals underwent three sessions of extinction in which animals were placed in the context 24, 48, and 72 h later (T1–T3) for a 10-min unreinforced trial.

The duration of freezing was measured using an analog signal generated in response to animal movement, by means of a high-sensitivity weight-transducer system connected to the grid floor. The signal was transmitted through the load-cell unit to the software module for recording and analysis of immobility (i.e., freezing).

Collection of tissue samples for western blot analysis

Both adult and juvenile rats were decapitated either 15 min after fear conditioning or 15 min after extinction (Chen et al. 2005).

Following decapitation, brains were quickly excised and snap-frozen in liquid nitrogen and stored at −80°C. The brains were then sliced on a cryostat (Leica CM1900, Germany). Following either fear conditioning or fear and extinction, samples of the BLA and the mPFC from the same animal were taken and were collected using a tissue curer 1 mm in diameter (FST, Germany). These samples were homogenized using a Glass-Teflon homogenizer in lysis buffer (10 mM HEPES, pH 7.6, 2 mM EDTA, 2 mM EGTA, pH 8.0, 0.5 mM DTT, 0.025% SDS, phosphatase and protease inhibitor cocktails [Sigma]). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The samples were diluted in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, and 5% β-mercaptoethanol), and then placed in 100°C for 5 min and stored at −20°C.

Western blotting

Sample with equal total protein concentration (10 μg) was loaded into Criterion TGX Gels, 4%–20%, 26-well (Bio-Rad). After standard electrophoresis the proteins were transferred to a nitrocellulose membrane (0.45 μm; Whatman) and proteins were visualized using Ponceau staining (Bio-Rad). Membranes were blocked for 1 h at room temperature with blocking buffer (5% blotting milk in TBS containing 0.1% Tween 20 [TBS-T]). Membranes were reacted with the primary antibody—Akt (1:1000), P-Akt (1:250) (Ser473, Cell Signaling Technology), or Actin (1:1000, Millipore) overnight at 4°C. Following three washing steps in TBS-T, the membranes were incubated with corresponding anti-rabbit (Jackson ImmunoResearch) or anti-goat (Millipore) secondary antibody. Proteins were visualized by enhanced chemiluminescense (ECL western blotting analysis system; GE Healthcare) and quantified with a charge-coupled device camera (XRS; Bio-Rad). Each sample was measured relative to the background, and phosphorylation levels were estimated by the ratio of phosphorylated to total protein.

Microinjection

Microinjection into the BLA or the IL-mPFC was performed through a 28-gauge injection needle placed in guide cannulae after the removal of the stylus. The injection needle was connected via PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (Harvard PHD 2000). PI3K inhibitor or vehicle (0.5 μL) was injected into each hemisphere over 1 min. The injection needle remained in the guide cannula for an additional minute to minimize fluid retraction.

Pharmacology

The cell-permeable PI3K-specific inhibitor, LY294002 (2-(4-morphlinyl)-8-phenyl-4*H*-1-benzopyran-4-one) (Sigma-Aldrich) (Vlahos et al. 1994; Barros et al. 2001; Horwood et al. 2006; Kritman and Maroun 2012; Slouzkey et al. 2013) (10 mM solution in DMSO) was dissolved in saline and brought to a final concentration of 25 μM. Control rats were injected with vehicle (DMSO in saline).

Statistical analysis

Differences were determined using mixed ANOVA followed by one-way ANOVA or Student's *t*-test. All post hoc comparisons were made using least significant difference (LSD) multiple comparison tests. All tests were two tailed, and a *P*-value of <0.05 was considered statistically significant. All tests were performed with SPSS version 17.0 (SPSS Inc.).

Competing interest statement

All authors reported no biomedical financial interests or potential conflicts of interest.

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