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AKT/GSK-3β/β-CATENIN SIGNALLING WITHIN HIPPOCAMPUS AND AMYGDALA REFLECTS GENETICALLY DETERMINED DIFFERENCES IN POSTTRAUMATIC STRESS DISORDER LIKE SYMPTOMS

M. DAHLHOFF,[a,b] A. SIEGMUND,[a1] Y. GOLUB,[a,c] E. WOLF,[b,d] F. HOLSBOER[a] AND C. T. WOTJAK[a]*

[a]*Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, D-80804 Munich, Germany*

[b]*Institute of Molecular Animal Breeding and Biotechnology, Gene Center, LMU Feodor-Lynen-Strasse 25, D-81377 Munich, Germany*

[c]*Graduate School of Neural and Behavioural Sciences, International Max Planck Research School, Tübingen, Germany*

[d]*Laboratory for Functional Genome Analysis, Gene Center, LMU Feodor-Lynen-Strasse 25, D-81377 Munich, Germany*

Abstract—Only a small percentage of individuals develop posttraumatic stress disorder (PTSD) in the aftermath of a trauma. It is still largely unknown to what extent gene-environment interactions contribute to the inter-individual differences in PTSD susceptibility and resilience and what cellular processes may underlie long-term maintenance of the disorder. Here we employed a mouse model of PTSD to unravel the contribution of genetic background and maternal influences on long-lasting changes in kinase and transcription factor activities in PTSD-susceptible C57BL/6NCrl (B6N) and resilient C57BL/6JOlaHsd (B6JOla) mice. Mice received an inescapable foot shock and were tested for activity changes in the AKT/GSK-3β/β-catenin-pathway in specific brain structures 42 days later. To control for prenatal and postnatal environmental (i.e. maternal) factors part of the experiments were performed with animals originating from within-strain and between-strain embryo transfers. In PTSD-susceptible B6N mice, long-term maintenance of contextual and sensitized fear was accompanied by (i) increased levels of phosphorylated AKT within the dorsal hippocampus and (ii) higher levels of phosphorylated AKT and GSK-3β and increased β-catenin levels within the basolateral amygdala. In animals originating from embryo transfers, levels of phosphorylated GSK-3β and of β-catenin were decreased in the dorsal hippocampus, but increased in the basolateral amygdala of shocked B6N mice compared to shocked B6JOla mice. This was independent of the genotype of the recipient mothers. At the behavioural level, these differences coincided with sustained sensitized and more pronounced contextual fear of B6N compared to B6JOla mice. Taken together our study identifies lasting changes in the AKT/GSK-3β/β-catenin cascade within the hippocampus and amygdala as molecular correlates of genetically determined differences in the severity of PTSD-like symptoms.

Key words: GSK-3β, β-catenin, nongenomic, contextual fear, sensitization, gene-environment interaction.

Individuals display heterogeneous responses to traumatic experiences, including resilience, rapid recovery or development of psychopathology such as posttraumatic stress disorder (PTSD) (Copeland et al., 2007; Kessler et al., 1995). In the latter case, exaggerated fear responses, avoidance behaviour, hyperarousal, and emotional numbing may persist for many years (Foa et al., 2006). One third of the affected patients maintain PTSD for 10 years or more despite therapeutic interventions (Kessler et al., 1995). The reasons for resilience or susceptibility to PTSD and long-term maintenance of the disease are still unclear. Evidence is accumulating that both genetic and environmental factors may shape individual vulnerability (Afifi et al., 2010; Galea et al., 2006; Betancourt and Khan, 2008). The lack of controllability of both genetic complexity and environmental factors in human populations asks for simpler models for dissecting the contributions of genetic vs. environmental factors to development of PTSD.

We recently established a mouse model of PTSD, which is well suited for addressing gene vs. environment interactions under controlled laboratory conditions (Siegmund and Wotjak, 2007a). This model is based on the exposure to a brief inescapable electric foot shock and fulfills all major criteria of an animal model of this disorder (Yehuda and Antelman, 1993; Siegmund and Wotjak, 2006). First, mice show persistent and exaggerated trauma-related fear, such as contextual fear memory that lasts for up to 7 months (Siegmund et al., 2009b) as well as hyperarousal and emotional numbing (Siegmund and Wotjak, 2007a). Second, behavioural symptoms can be reversed by chronic SSRI treatment (Siegmund and Wotjak, 2007b). Third, we observed an inverse relationship between hippocampal N-acetylasparate (NAA) concentrations before trauma and severity and persistence of PTSD-like symptoms (Siegmund et al., 2009b).

Experiments revealed a remarkable variance in the behavioural and molecular measures despite the fact that studies had been performed with inbred, that is genetically identical, mice. Consequently, we looked for environmental factors, which may influence individual susceptibility for developing PTSD-like symptoms and identified maternal inexperience as a potential risk factor (Siegmund et al., 2009a). However, the genotype of the animals also seems to play a significant role, since C57BL/6NCrl (B6N) mice showed considerably more pronounced contextual and

[1] Present address: Clinic of Psychiatry and Psychotherapy, Campus Charité Mitte, Berlin, Germany.

*Corresponding author: Tel: +49-89-30622-652; fax: +49-89-30622-610.
E-mail address: wotjak@mpipsykl.mpg.de (C. T. Wotjak).

Abbreviations: BSA, bovine serum albumin; PSTD, posttraumatic stress disorder.

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sensitised fear than mice of a closely related inbred strain (C57BL/6JOlaHsd, B6JOla) 1 month after application of the foot shock (Siegmund and Wotjak, 2007a; Siegmund et al., 2009a). Interestingly, the effects of maternal inexperience on PTSD susceptibility were restricted to the B6N strain (Siegmund et al., 2009a).

Little is known about molecular correlates of inter-individual differences in long-term maintenance and severity of PTSD-like symptoms. Some authors reported a potential involvement of changes in expression, of the brain-derived neurotrophic factor (BDNF; Kozlovsky et al., 2007; Krishnan et al., 2007) and adenylate cyclase activity and of recurrent activation of N-methyl-D-aspartate (NMDA) receptors (Cui et al., 2004). In addition, kinases and transcription factors are known to affect consolidation of aversive memories, at least in the early aftermath of an aversive encounter. For instance, extracellular regulated kinases (ERK or mitogen-activated protein kinases, MAPK) and protein kinase B (PKB, AKT) mediate consolidation of fear memories at 60 min after fear conditioning (Ahi et al., 2004). The same applies to their potential down-stream target, cAMP-response element binding protein (CREB) (Trifilieff et al., 2006), which controls gene transcription. A recent publication suggests that the transcription factor β-catenin plays a similarly important role in consolidation of fear memories at the level of the basolateral amygdala (Maguschak and Ressler, 2008; Gould et al., 2008). One should note that β-catenin is under control of glycogen synthase kinase-3β (GSK-3β). Under basal conditions, GSK-3β is constitutively active and destabilizes β-catenin by phosphorylation, which leads to its proteasomal degradation (Gould et al., 2008; Wada, 2009). However, GSK-3β can be inactivated by phosphorylation of the Ser 9 residue through phosphorylated AKT. Phosphorylation of GSK-3β stabilizes β-catenin, which can then migrate into the nucleus and mediate gene transcription for example, of *Bdnf, Vegf*, *Igf 1* and *Igf 2* (Wada, 2009).

Despite the plethora of evidence for a role of kinases and transcription factors in early processing of aversive events, there are very few reports about activity changes, which outlast the encounter for more than a couple of hours (Krishnan et al., 2007). Therefore, we decided to study AKT/GSK-3β/β-catenin-cascades within the dorsal hippocampus and basolateral amygdala in the long-term aftermath of exposure to an inescapable foot shock. Both brain structures are known to undergo long-lasting changes in human PTSD (Bremner et al., 2008) as well as in established animal models of PTSD (Krishnan et al., 2007; Huang and Kandel, 2007; Trifilieff et al., 2007), and seem to be causally involved in PTSD-related symptoms (Kozlovsky et al., 2007). To relate differences in kinase activity and the intensity of PTSD-like symptoms to genetic and/or environmental factors, we analysed behavioural performance (i.e. sensitized and contextual fear) and intracellular signalling cascades in PTSD-susceptible B6N and PTSD-resilient B6JOla mice originating from within- and between-strain embryo transfers.

EXPERIMENTAL PROCEDURES

Subjects

Male and female C57BL/6NCrl (B6N; obtained from Charles River Laboratories; Sulzfeld, Germany) and C57BL/6JOlaHsd (B6JOla; obtained from Harlan-Winkelmann; Borchen, Germany) mice were maintained under specific pathogen-free conditions in the closed barrier facility of the Gene Center Munich at 23 °C, 40% humidity and with a 12-h light/dark cycle (lights on at 7 AM), and had free access to a standard rodent diet (V1534, Ssniff, Soest, Germany) and water. Embryo-transfers were performed at the Gene Center. At an age of 6 weeks, male offspring were transferred to the mouse facility of the Max Planck Institute of Psychiatry for behavioural analyses. All experiments were approved by the Committee on Animal Health and Care of the local governmental body of the state of Upper Bavaria (Regierung von Oberbayern; Az 55.2-1-54-2531-14-03) and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals (European Communities Council Directive of November 24, 1986 [86/609/EEC]).

Embryo transfer and housing of animals

To produce blastocysts for the embryo transfer experiment, 8-week-old females (donors) from both strains were mated with males of the same mouse strain. Females were screened for vaginal plugs every morning and evening and sacrificed by cervical dislocation at day 3 after finding a vaginal plug (3.5 dpc). The uterus was removed and flushed with M2 medium containing 0.4% bovine serum albumin (BSA). Blastocysts were collected under a stereomicroscope with 20× magnification (Nagy et al., 2003) and transferred to microdrops of M2 medium with 0.4% BSA on a culture dish covered with paraffin oil at 37 °C until needed. Between 12 and 20 embryos were transferred into the uteri of pseudopregnant female B6N or B6JOla mice (recipients) that were prepared by mating 12-week-old females with vasectomized males. These females were also screened for vaginal plugs every morning and used as recipients for the blastocysts at day 2 (2.5 dpc) after finding a vaginal plug. The skin and muscles of the back of the anesthetized recipient were cut and the uterine horns were externalized from the peritoneal cavity. Under a stereomicroscope with 20× magnification the uterus was punched with a needle near the oviduct. A transfer pipette (loaded with embryos in M2 medium) was inserted through the punched hole, and the embryos were placed into the uterus. Blastocysts of each mouse strain were transferred to recipients of the same or the other strain, depending on the experimental group. Pregnant recipients were housed in Makrolon type II long cages (36×21×12 cm^3) and monitored for birth. All recipients gave birth normally. A total of seven litters (n=23♂/22♀) from B6N donors and B6N recipients (N/N), five litters (n=17♂/15♀) from B6N donors and B6JOla recipients (N/J), six litters (n=16♂/16♀) from B6JOla donors and B6JOla recipients (J/J), and four litters (n=11♂/10♀) from B6JOla donors and B6N recipients (J/N) were born. Within 12 h of birth [postnatal day (pnd) 0] litters with more than 10 pups were reduced to 10 animals per litter by discarding surplus pups. Thereafter, animals remained undisturbed until behavioural tests started.

Offspring were weaned at day 31 and housed in groups of siblings with a maximum of four animals in Makrolon type III cages (42×26×14 cm^3) containing a running wheel, a transparent plastic tube and cellulose. At the age of 6 weeks, male offspring were transferred to the mouse facility of the Max Planck Institute of Psychiatry and the groups were housed in Makrolon type II cages (27×16×12 cm^3) containing wood shavings (Altromin Faser Einstreu, Altromin GmbH, Germany) and cellulose as nest material. Mice were acclimatized for 1 week to the new environment and the inverse 12 h:12 h light/dark cycle (lights on at 9 PM), before they were separated and housed singly and acclimatized for another

10–14 days before starting the experiments. Mice were maintained under standard conditions with food (Altromin Standard Diet 1310, Altromin GmbH, Germany) and tap water *ad libitum*.

Shock application and test of conditioned and sensitized fear

Experimental set-up and experimental design have been described in detail before (Siegmund and Wotjak, 2007a; Kamprath and Wotjak, 2004). Briefly, animals were placed in a transparent cubic-shaped chamber with a metal grid for shock application. After 198 s a single electric foot shock (1.5 mA) of 2 s duration was administered via the metal grid. Mice were returned to their cages after another 60 s. Non-shocked control animals were not exposed to the conditioning chamber. Animals were left undisturbed for 4 weeks. In the morning of day 28 after shock application, conditioned fear was measured by placing the animals back to the shock context for 180 s. In the afternoon, sensitized fear was tested by exposing the mice to a 180 s continuous tone (80 dB, 9 kHz) in a neutral, transparent, cylindrical chamber. Each session was videotaped for subsequent off-line analysis of freezing behaviour.

Isolation of brain regions

Two weeks after completion of behavioural testing, mice were anesthetized with isoflurane (DeltaSelect, Germany) and rapidly killed. To minimize stress levels and unspecific context reminders, mice were individually transferred to the neighboring room, anesthetized with isoflurane followed by cervical dislocation, lasting in toto not longer than 2–3 min. Brains were extracted on ice, snap-frozen and kept at −80 °C. For brain dissection, brains were cut with a cryostat (Microm, Walldorf, Germany) up to the appearance of the brain region of interest. Brain specimens of the respective brain region were isolated using cylindrical punchers (Fine Science Tools, Heidelberg, Germany). The location and length of the punches were chosen on basis of a stereotaxic atlas (Paxinos and Franklin, 2001) as follows:

(1) Dorsal hippocampus: Starting at 1.8 mm posterior to bregma, with a diameter of 1.0 mm and a punch-length of 1.0 mm in order to collect the CA1 region of the dorsal hippocampus including the dentate gyrus and a part of the CA3 region (cf. Fig. 7A);
(2) Amygdala: Starting at 0.8 mm posterior to bregma, with a diameter of 1.0 mm and a punch-length of 1.0 mm in order to collect the anterior part of the basolateral amygdaloid nucleus and lateral amygdaloid nucleus (cf. Fig. 8A).

Punches from both brain hemispheres of each region were pooled per animal and stored at −80 °C until processing for Western blot analyses. The dissection site was verified by histological analyses using a stereomicroscope.

Western blot analysis

For protein extraction, the brain tissue was homogenized in Laemmli-extraction-buffer, and the protein content was estimated by the bicinchoninic acid (BCA) protein assay. 20 μg of total protein were separated by 12% SDS-PAGE and transferred to PDVF membranes (Millipore, Billerica, USA) by electroblotting. Membranes were washed in Tris-buffered saline solution with 0.1% Tween 20 (TBS-T; Sigma, Deisenhofen, Germany) and blocked in 5% w/v fat-free milk powder (Roth, Karlsruhe, Germany) for 1 h at room temperature. Then membranes were washed again in TBS-T and incubated in 5% w/v BSA (Sigma) of the appropriate primary antibody overnight at 4 °C. As primary antibodies we used rabbit polyclonal anti-phospho-AKT (Ser 473; 1:2000; #9271; Cell Signaling, Ipswich, USA), rabbit polyclonal anti-phospho-GSK-3β (Ser 9; 1:2000; #9336; Cell Signaling) and mouse monoclonal anti-β-catenin (1:5000; # 610154; BD Transduction Laboratories, Heidelberg, Germany). Henceforward we will use the term "pProtein" instead of "phospho-Protein" for the sake of brevity. After washing, membranes were incubated in 5% w/v fat-free milk powder with a horseradish peroxidase labelled secondary antibody (donkey anti-rabbit; 1:2000; NA934V; GE-Healthcare, Munich, Germany or rabbit anti mouse; 1:2000; #7076; Cell Signaling) for 1 h at room temperature. Bound antibodies were detected using an enhanced chemiluminescence detection reagent (ECL Advance Western Blotting Detection Kit, GE Healthcare) and appropriate x-ray films (GE Healthcare). After detection, membranes were stripped (Elution buffer: 2% SDS, 62.5 mM Tris–HCl, pH 6.7 and 100 mM β-mercaptoethanol for 40 min at 70 °C) and incubated with a second antibody recognizing total AKT and GSK-3β as described before by using rabbit polyclonal anti-AKT (1:2000; #9272; Cell Signaling), rabbit polyclonal anti-GSK-3β (1:2000; #9318; Cell Signaling) and rabbit polyclonal anti-GAPDH (1:5000; #2118; Cell Signaling). Western blot membranes of phosphorylated proteins were stripped again and incubated with a second antibody recognizing GAPDH as a control for changes in kinase expression irrespective of the activity status. Band intensities were quantified using the ImageQuant software package (GE Healthcare).

Experiments

Experiment 1. Male B6N mice purchased from Charles River were randomly assigned to one out of two groups. One group received the foot shock while the other remained unshocked. All animals were tested for sensitized and contextual fear (28 days after shock) before brain removal (42 days after shock).

Experiment 2. Male B6N and B6JOla mice originating from within-strain (N/N, J/J) and between-strain (N/J, J/N) embryo transfers were shocked, phenotyped and the respective brain structures were processed for Western blot analyses essentially as described in Experiment 1. The limited yield of the transfers did not allow inclusion of unshocked controls.

Data analysis and statistics

Freezing behaviour was defined as immobility except for respiration movements and analysed off-line by trained observers, who were blind to the conditioning procedure and mouse strain, and quantified by means of customized software (EVENTLOG, Henderson, 1986) as described (Kamprath and Wotjak, 2004). Sensitized and contextual fear were analysed in 20-s bins by 2-way or 3-way ANOVAs as described in the text, followed by Newman–Keuls post hoc test, if appropriate, using Statistica 5.0 (StatSoft, Tusla, OK, USA).

For measurement of kinases and transcription factor activities, pAKT, pGSK-3β, and β-Catenin bands were densitometrically analysed followed by normalization to the corresponding total AKT, GSK-3β or GAPDH levels. To control for general changes in kinase expression, we additionally normalized total AKT and total GSK-3β levels to GAPDH. Comparisons were performed only for data obtained from the same blot, thus resulting in two-group comparisons with $n=7$ per group. Specimens were repeatedly blotted from stock in case of multiple comparisons with other groups. For each blot, all values were related to the mean value of the respective reference group (non-shocked B6N or groups where the donors and/or the recipients were B6JOla mice) and compared by Mann–Whitney U-tests (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA).

Data are presented as means±SEM (behaviour) or box-plots with median (Western blot). Group difference were considered to be statistically significant if $P<0.05$.

RESULTS

PTSD-like symptoms coincide with changes in kinase and transcription factor activities at remote time points

Shocked B6N animals from experiment 1 showed high levels of contextual (i.e. freezing to the shock context) and sensitized fear (i.e. freezing to the tone) compared to non-shocked controls 28 days after foot shock (Fig. 1C). Western blot analyses revealed significantly increased levels of pAKT ($P=0.007$) in the hippocampus, but no differences in pGSK-3β ($P=0.456$) and β-catenin ($P=0.165$), in shocked vs. non-shocked mice 42 days after the aversive encounter (Fig. 1A). In the amygdala, levels of pAKT ($P=0.030$), pGSK-3β ($P=0.0006$) and β-catenin ($P=0.001$) were significantly increased (Fig. 1B). Expression levels of total AKT and total GSK-3β, as assessed after normalization to GAPDH, remained unchanged (data not shown).

Genetic and maternal effects on PTSD-like symptoms

Next we ought to relate the changes in kinase activity to the severity of PTSD-like symptoms on basis of strain differences in the mouse model of PTSD (Siegmund and Wotjak, 2007a; Siegmund et al., 2009a). Behavioral analysis of non-shocked and shocked B6N and B6JOla mice obtained from the commercial breeders confirmed the higher susceptibility of the former strain, with B6N showing more contextual fear [strain×shock: $F_{1,41}=15.91$, $P<0.0005$; 3-way ANOVAs (strain, shock, interval) for repeated measures (interval)] and sustained sensitized fear [strain×shock: $F_{1,41}=4.12$, $P=0.049$; strain×shock×interval: $F_{8,328}=2.27$, $P=0.022$] compared to shocked B6JOla (Fig. 2A).

To differentiate between maternal and genetic differences in the development of PTSD-like symptoms, we performed embryo transfers within and between strains and compared the behavioural characteristics of the offspring with respect to genotypic (factor "genotype"/"donor") and pre-/postnatal environment (factor "recipient mother") influences.

Analysis of contextual fear by 3-way ANOVA (genotype, recipient mother, interval) for repeated measures (interval) revealed significant main effects of genotype ($F_{1,60}=7.894$, $P=0.007$) and recipient mother ($F_{1,60}=4.777$, $P=0.033$) but no significant genotype×recipient mother ($F_{1,60}=0.048$, $P=0.827$) or genotype×recipient mother×interval ($F_{8,480}=1.863$, $P=0.064$) interactions, indicating (i) that B6N offspring showed elevated levels of freezing compared to B6JOla offspring, irrespective of the genotype of the recipient mothers (genetic effect), and (ii) that offspring born by B6JOla mothers showed generally higher levels of contextual fear compared to offspring of the same genotype born by B6N mothers (maternal effect) (Fig. 2B).

Analysis of sensitized fear revealed significant genotype×interval ($F_{8,512}=5.425$, $P<0.0001$) and recipient mother×interval ($F_{8,512}=2.589$, $P=0.009$), but no genotype×recipient mother×interval ($F_{8,512}=0.5949$, $P=0.783$) or genotype×recipient mother interactions ($F_{1,64}=0.002$, $P=0.961$), indicating that (i) B6N offspring showed elevated levels of freezing towards the end of tone presentation, whereas B6JOla offspring showed fear adaptation, irrespective of the genotype of the recipient mothers (ge-

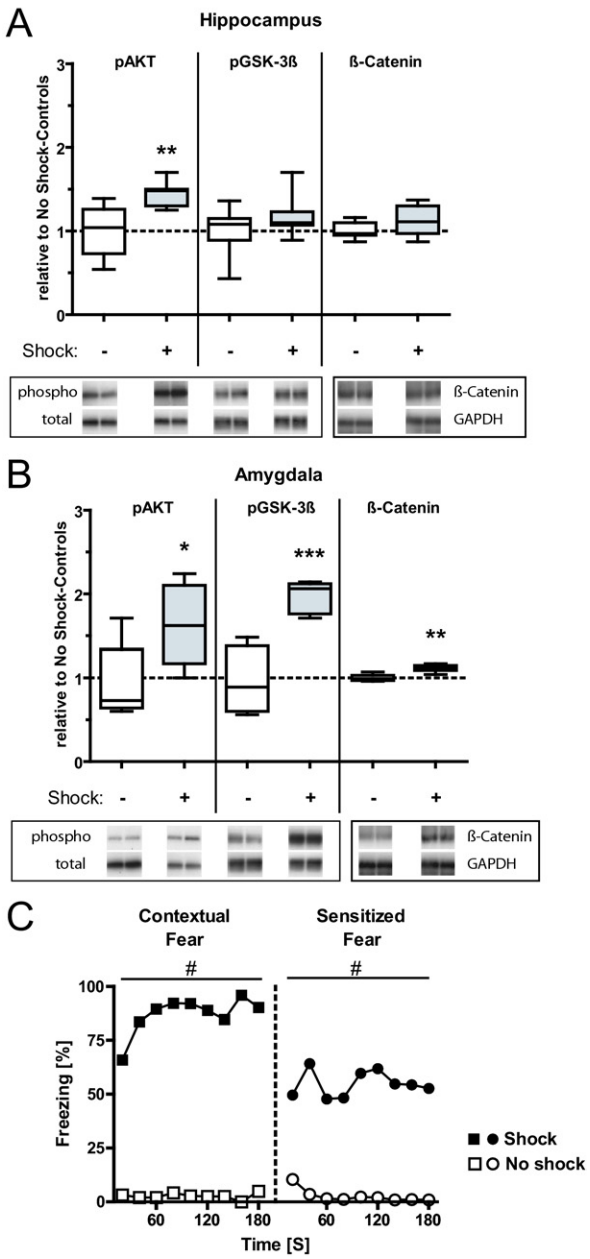

Fig. 1. Kinase and transcription factor activities in shocked vs. non-shocked B6N mice. Western blot analyses of phosphorylated kinases and transcription factors in brain punches obtained from (A) Hippocampus and (B) Amygdala of shocked and non-shocked B6N mice (purchased from the commercial breeders) 42 days after foot shock application. Levels of phosphorylated protein were quantified densitometrically, normalized to total levels of the phosphorylated/non-phosphorylated protein (pAKT/AKT and pGSK-3β/GSK-3β) or GAPDH levels (β-catenin/GAPDH) and are expressed relative to the mean levels of non-shocked controls. Data are presented as box/whisker blots ($n=7$ per group). *$P<0.05$, ** $P<0.01$, *** $P<0.001$ (Mann–Whitney U-test). (C) Line graphs show the temporal development of contextual and sensitized fear of shocked and non-shocked B6N mice. # $P<0.01$ vs. non-shocked group (2-way ANOVA).

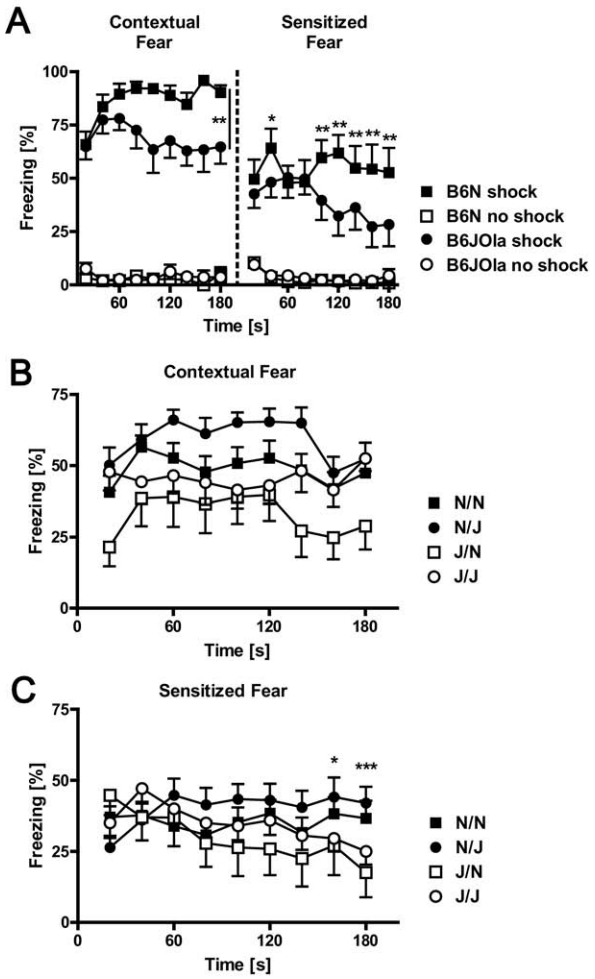

Fig. 2. Fear behaviour in mice originating from commercial breeders or within-strain and between-strain embryo transfers. (A) Freezing responses of shocked and non-shocked B6N mice and B6JOla obtained from commercial breeders upon re-exposure to the conditioning context (i.e. contextual fear) and to a neutral tone in a neutral environment (i.e. sensitized fear) 28 days after shock application (n=10–12 per group). (B, C) Contextual and sensitized fear of B6N (N) and B6JOla mice (J) derived from within-strain (N/N, J/J) and between-strain (N/J, J/N) embryo transfers (n=11–23 per group; donor strain/recipient strain). Mean±SEM, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. B6JOla offspring (3-way ANOVAs followed by Newman–Keuls post hoc test).

netic effect) and (ii) offspring born by B6JOla mothers showed higher levels of sensitized fear compared to offspring of the same genotype born by B6N mothers (maternal effect) (Fig. 2C).

Genetic contribution to changes in kinase and transcription factor activities

To assess the contribution of the genotype of the animals to strain differences in kinase and transcription factor activities, we compared brain specimens of B6N and B6JOla mice transferred to the same recipient strain (thereby keeping the factor "maternal influence" constant). In the hippocampus, pGSK-3β (P=0.0012) and β-catenin (P=0.0175), but not pAKT (P=0.1649), were significantly decreased in B6N mice transferred to B6N mothers (N/N) compared to B6JOla mice transferred to B6N mothers (J/N; Fig. 3A). In the amygdala, the levels of pAKT (P=0.007), pGSK-3β (P=0.0006) and β-catenin (P=0.001) were significantly increased in N/N-compared to J/N-offspring (Fig. 3B). Comparison of PTSD-like symptoms revealed that the differences in kinase and transcription factor activity coincided with a trend towards increased contextual fear (genotype: $F_{1,29}$=3.46, P=0.073) and significantly increased sensitized fear in N/N compared to J/N offspring towards the end of tone presentation (genotype×time: $F_{8,264}$=2.39, P=0.017; Fig. 3C).

In hippocampus specimens of offspring raised by B6JOla recipients, pGSK-3β (P=0.0006) and β-catenin (P=0.0006) were significantly decreased in N/J compared to J/J offspring with no differences in pAKT (P=0.318) (Fig. 4A). In the amygdala the levels of pAKT (P=0.0012), pGSK-3β (P=0.007) and β-catenin (P=0.038) were significantly increased in N/J compared to J/J offspring (Fig. 4B). The differences in kinase and transcription factor activities coincided with increased contextual fear (genotype: $F_{1,31}$=4.60, P=0.04; genotype×time: $F_{8,248}$=2.37, P=0.018) and increased sensitized fear in N/J compared to J/J offspring towards the end of tone presentation (genotype×time: $F_{8,248}$=3.75, $P<0.001$; Fig. 4C).

Maternal contribution to changes in kinase and transcription factor activities

To assess the contribution of maternal factors to the strain differences in kinase and transcription factor activities, we compared brain specimens of B6N and B6JOla mice, respectively, transferred to different recipient strains. We failed to detect significant differences in phosphorylation levels in hippocampus and amygdala of B6N mice raised by B6N recipients (N/N) compared to B6N raised by B6JOla recipients (N/J) (statistics not shown; Fig. 5A, B). This coincided with only small and/or non-significant differences in contextual (recipient strain: P=0.116; recipient strain×time: P=0.359) and sensitized fear (recipient strain: P=0.476; recipient strain×time: P=0.036) and with both groups of mice showing a non-decaying freezing response to the tone (Fig. 5C).

B6JOla mice transferred to B6N recipients (J/N) showed higher levels of pAKT in hippocampus (P=0.011) and amygdala (P=0.0006) compared to B6JOla mice transferred to B6JOla recipients (J/J) (Fig. 6A, B). However, other than for all the other analyses where total kinase levels normalized to GAPDH were similar between the groups (data not shown), this time total AKT levels were significantly decreased in J/N compared to J/J (0.35±0.04 vs. 1.0±0.19; P=0.0057). Therefore, the increase in pAKT relative to total AKT has to be interpreted with caution, since it may represent a mathematical phenomenon and not a general increase in pAKT levels. In contrast, pGSK-3β was significantly reduced in the amygdala (Fig. 6B) with no changes in total GSK-3β. At the behavioural level, there were no significant differences in freezing behaviour (contextual fear: recipient strain: P=0.149; recipient strain×time: P=0.143; sensitized fear:

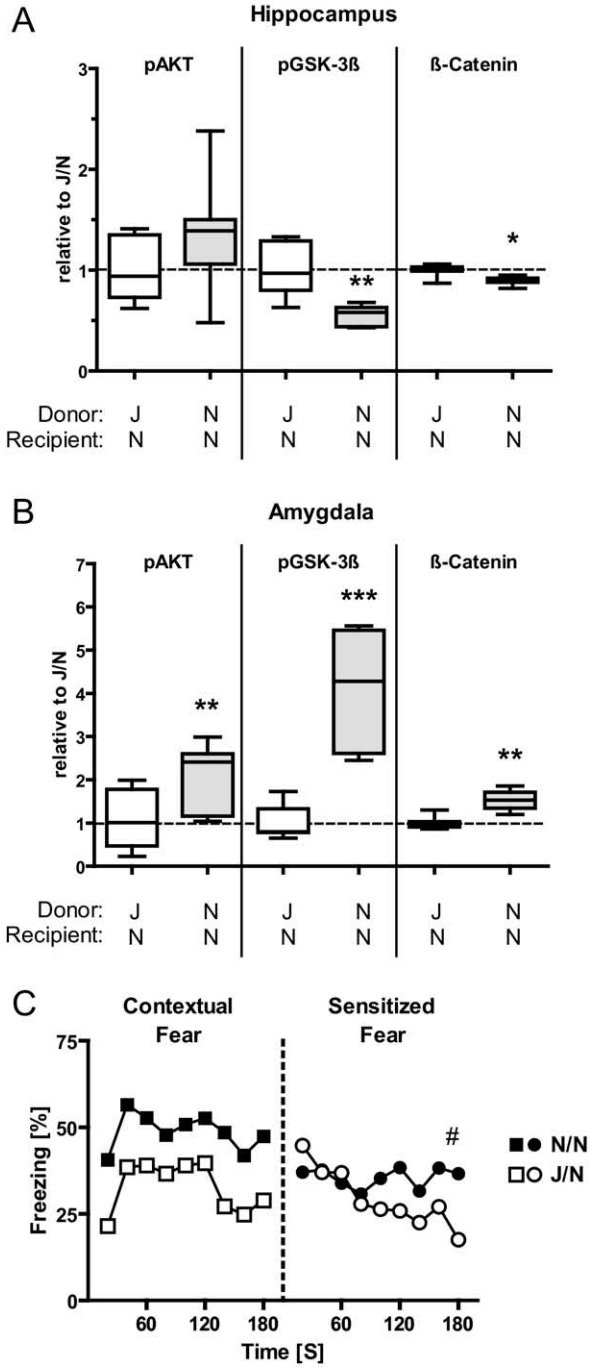

Fig. 3. Kinase and transcription factor activities in B6N and B6JOla mice transferred to the B6N strain (donor-effect). Western blot analyses of phosphorylated kinases and transcription factors in brain punches obtained from (A) Hippocampus and (B) Amygdala of shocked N/N and J/N mice 42 days after foot shock application. Levels of phosphorylated protein were quantified densitometrically, normalized to total levels of the phosphorylated/non-phosphorylated protein or GAPDH levels and expressed relative to the mean levels of J/N mice. Data are presented as box/whisker blots ($n=7$ per group); * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. other group (Mann–Whitney U-test). (C) Line graphs (means) show the temporal development of contextual and sensitized fear of the animals under study (data from Fig. 2B, C). # $P<0.05$ (significant genotype×time interaction in the 2-way ANOVA followed by Newman–Keuls post hoc test).

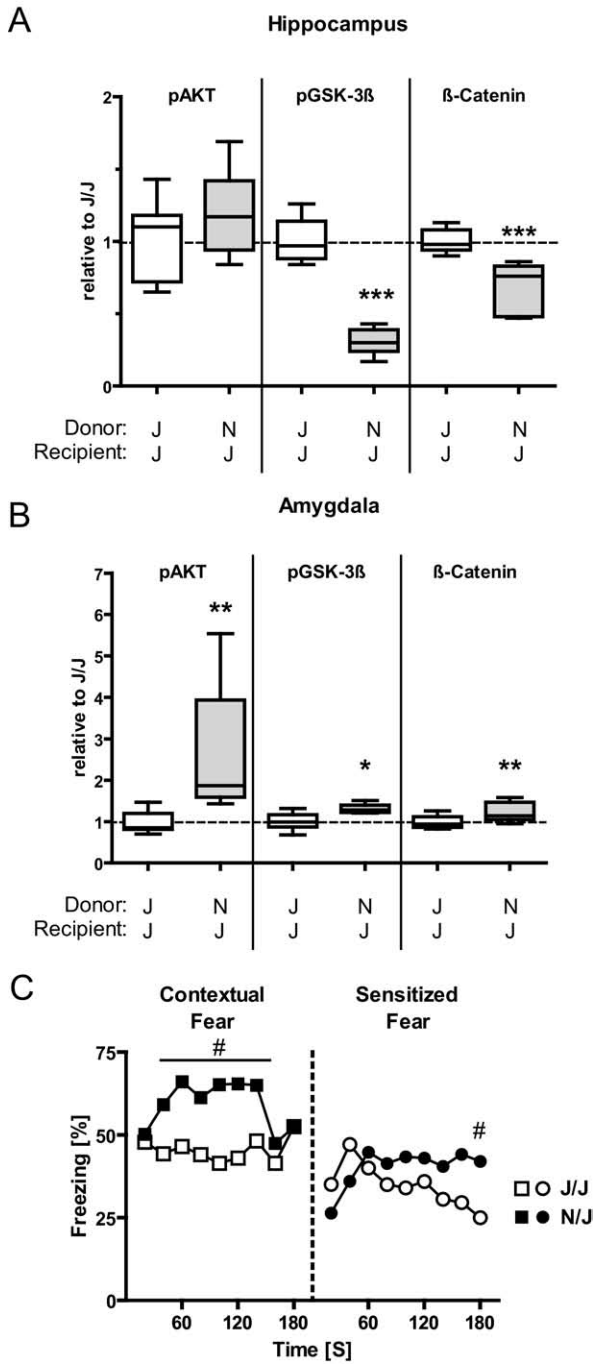

Fig. 4. Kinase and transcription factor activities of B6N and B6JOla mice transferred to the B6JOla strain (donor-effect). Western blot analyses of phosphorylated kinases and transcription factors in brain punches obtained from (A) Hippocampus and (B) Amygdala of shocked N/J and J/J mice 42 days after foot shock application. Levels of phosphorylated protein were quantified densitometrically, normalized to total levels of the phosphorylated/non-phosphorylated protein or GAPDH levels and expressed relative to the mean levels of J/J mice. Data are presented as box/whisker blots ($n=7$ per group); * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. other group (Mann–Whitney U-test). (C) Line graphs (means) show the temporal development of contextual and sensitized fear of the animals under study (data from Fig. 2B, C). # $P<0.05$ (significant genotype×time interaction in the 2-way ANOVA followed by Newman–Keuls post hoc test).

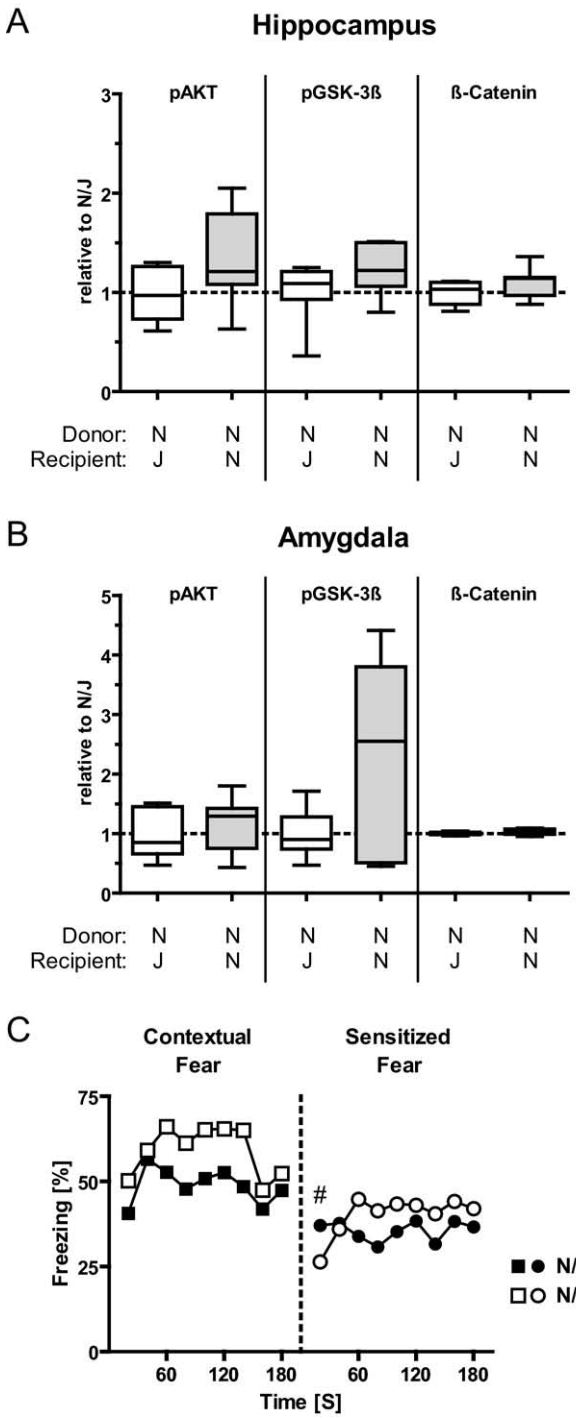

Fig. 5. Kinase and transcription factor activities of B6N mice transferred to the B6N and B6JOla strain (recipient-effect). Western blot analyses of phosphorylated kinases and transcription factors in brain punches obtained from (A) Hippocampus and (B) Amygdala of shocked N/N and N/J mice 42 days after foot shock application. Levels of phosphorylated protein were quantified densitometrically, normalized to total levels of the phosphorylated/non-phosphorylated protein or GAPDH levels and expressed relative to the mean levels of N/J mice. Data are presented as box/whisker blots ($n=7$ per group). (C) Line graphs (means) show the temporal development of contextual and sensitized fear of the animals under study (data from Fig. 2B, C). # $P<0.05$ (significant genotype×time interaction in the 2-way ANOVA followed by Newman–Keuls post hoc test).

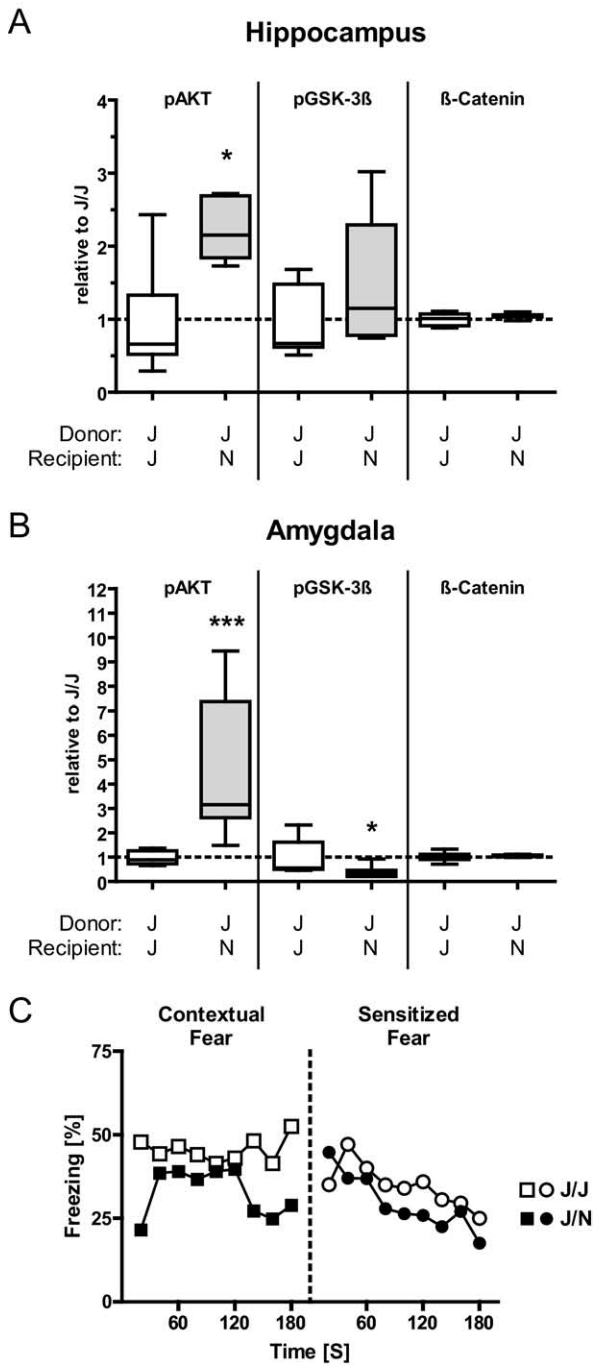

Fig. 6. Kinase and transcription factor activities of B6JOla mice transferred to the B6JOla and B6N strain (recipient-effect). Western blot analyses of phosphorylated kinases and transcription factors in brain punches obtained from (A) Hippocampus and (B) Amygdala of shocked J/N and J/J mice 42 days after foot shock application. Levels of phosphorylated protein were quantified densitometrically, normalized to total levels of the phosphorylated/non-phosphorylated protein or GAPDH levels and expressed relative to the mean levels of J/J mice. Data are presented as box/whisker blots ($n=7$ per group); * $P<0.05$, *** $P<0.001$ vs. other group (Mann–Whitney U-test). (C) Line graphs (means) show the temporal development of contextual and sensitized fear of the animals under study (data from Fig. 2B, C).

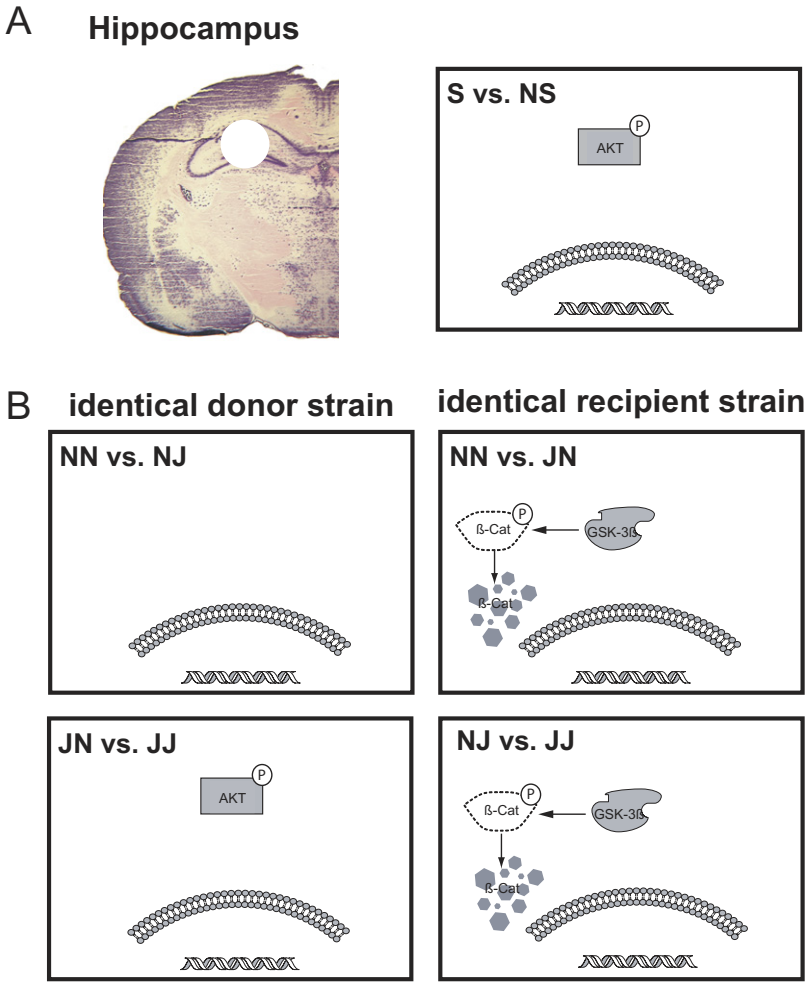

Fig. 7. Changes in signalling cascades within the hippocampus. Schemes translate the Western blot results of the hippocampus (Figs. 1A, 3A–6A) into hypothetical scenarios depicting changes in intracellular signalling cascades (filled symbols reflect changes measured by Western blot, open symbols with dotted lines stand for hypothetical changes) in shocked (S) vs. non-shocked (NS) B6N (A) and shocked offspring of within- and between-strain embryo transfers (B). In this context, higher phosphorylation levels were interpreted as increased pAKT respectively decreased pGSK-3β activity. Note that inactivation of GSK-3β by phosphorylation is known to result in stabilization of β-catenin. The white circle in the hematoxylin-eosin stained histological picture in panel (A) indicates the exact localization of the brain punches in the dorsal hippocampus.

recipient strain: $P=0.533$; recipient strain×time: $P=0.232$ Fig. 6C).

DISCUSSION

The present study demonstrates that long-term maintenance of PTSD-like symptoms in the PTSD-susceptible B6N strain coincides with changes in kinase activities within the hippocampus and amygdala, compared to non-shocked animals and to the PTSD-resilient B6JOla strain. As revealed by within-strain and between-strain embryo transfers, the strain differences at the behavioural level were determined by both genetic and maternal influences. In contrast, strain differences at the molecular level were strongly related to the genotype of the offspring irrespective of the genotype of the mother (Figs. 7B and 8B). This applies to the activity status of GSK-3β and its influence on β-catenin levels, with increased GSK-3β activity/decreased β-catenin levels in the hippocampus (Fig. 7B) and decreased GSK-3β activity/increased β-catenin levels in the amygdala (Fig. 8B) of PTSD-susceptible vs. PTSD-resilient strains under circumstances of sustained sensitized and increased contextual fear.

The changes in the intracellular signalling cascades were assessed 42 days after a single, brief electric foot shock and 2 weeks after behavioural testing. Our experimental design does not allow to dissect the exact contribution of each of those two processes. In addition, we don't know how soon they appear after the foot shock. Other studies have only reported transient changes in kinase activity in the early aftermath of a stressor exposure including fear conditioning (Di Benedetto et al., 2009; Paul et al., 2007) that returned to baseline within 1–2 days. It may be that there are different waves of kinase activity, if one considers that, with the passage of time, fear incubation also leads to qualitative changes in behavioural characteristics (Siegmund and Wotjak, 2007b; Balogh et al., 2002).

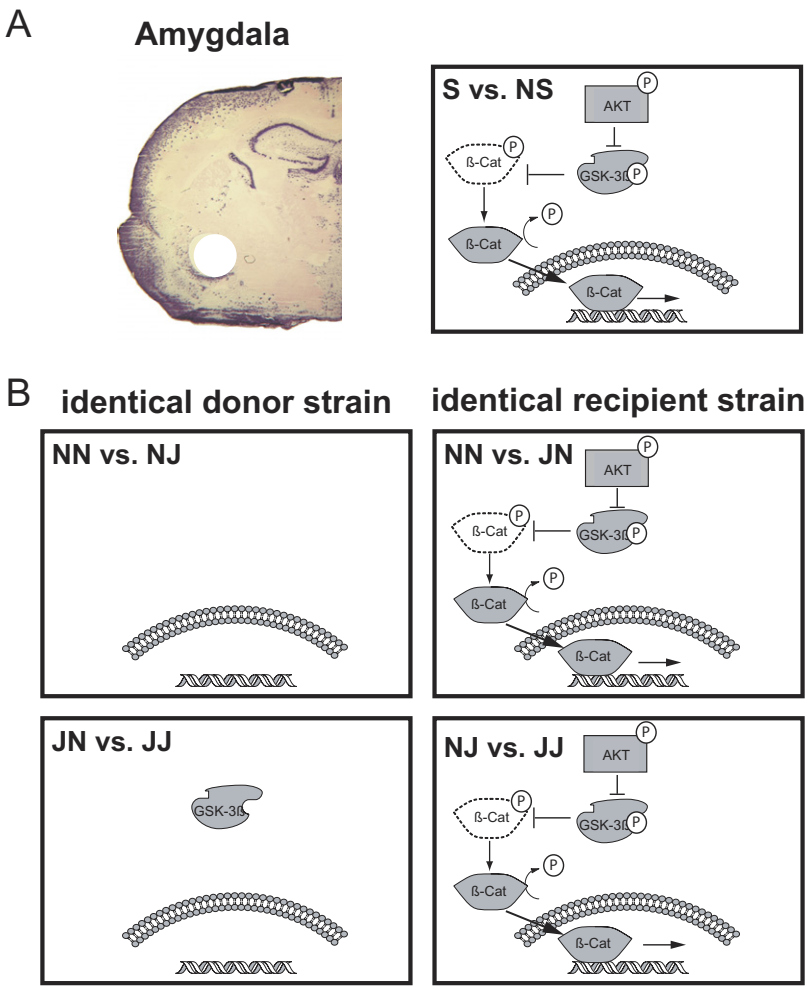

Fig. 8. Changes in signalling cascades within the amygdala. Schemes translate the Western blot results of the amygdala (Figs. 1B, 3B–6B) into hypothetical scenarios depicting changes in intracellular signalling cascades (filled symbols reflect changes measured by Western blot, open symbols with dotted lines stand for hypothetical changes) in shocked (S) vs. non-shocked (NS) B6N (A) and shocked offspring of within- and between-strain embryo transfers (B). In this context, higher phosphorylation levels were interpreted as increased pAKT respectively decreased pGSK-3β activity. Note that inactivation of GSK-3β by phosphorylation is known to result in stabilization of β-catenin. The increased levels of pAKT in J/N vs. J/J mice have to be interpreted with caution, since total AKT levels normalized to GAPDH were decreased in the same samples (for details see text). The white circle in the hematoxylin-eosin stained histological picture in panel (A) indicates the exact localization of the brain punches in the basolateral amygdala complex.

For example, it has been observed that there is a loss of specificity, but maintenance of intensity of contextual fear (Wiltgen and Silva, 2007). It is tempting to speculate that the changes in kinase activity observed in the present study contribute to this phenotype, similarly to the role of PKA/adenylate cyclase 1 (Shan et al., 2008), NMDA receptor NR1 subunits (Cui et al., 2004) and CaMKII (Frankland et al., 2001) in development and/or maintenance of remote contextual fear memory.

Both behavioural symptoms (Siegmund and Wotjak, 2007a; Siegmund et al., 2009a), and activity status of intracellular signalling cascades may depend on genotypic and environmental influences during early development. To differentiate between these two factors, we performed within-strain and between-strain embryo transfers. This allowed us to control for both prenatal and postnatal maternal influences, which have been shown to affect behavioural performance in an interactive manner (Francis et al., 2003). Results of our embryo transfers revealed that PTSD-like symptoms are determined by the genotype of the test animals. Unexpectedly, however, offspring of embryo transfers to mothers of the less PTSD-vulnerable strain showed higher levels of sensitized fear than respective transfers to the B6N strain (Fig. 2C), an effect which could not be statistically secured if between-strain transfers were separately analysed (Figs. 5C and 6C). Such effects as well as differences in freezing levels between B6N and B6JOla obtained from commercial breeders and those originating from embryo transfer experiments might be partially ascribed to strain differences in susceptibility to surgical stress, as recently reported for C3H/HeN and DBA/2J mice (Rose et al., 2006), and corroborate observations that surgical stress may translate into changes in behavioural performance of the offspring

(Suchecki and Palermo, 1991; Van den Hove et al., 2005, 2006).

Maternal effects on PTSD-like behaviour were small and showed little correspondence to changes in kinase and transcription factor activities (N/N vs. N/J, J/N vs. J/J; Figs. 7B and 8B). This observation is in line with other reports about resilience of B6 strains to maternal factors (Gleason et al., 2010; Millstein and Holmes, 2007). The genetic background of the offspring, in contrast, consistently revealed the same behavioural and molecular phenotypes, irrespective of the genotype of the recipient mothers. B6N and B6JOla originating from transfers to the same recipient strain (i.e. N/N vs. J/N, N/J vs. J/J) showed not only sustained vs. decaying sensitized fear, but also an activation of the AKT/GSK-3β/β-catenin pathway within the basolateral amygdala (Fig. 8B) and an inactivation of this signalling cascade within the hippocampus (Fig. 7B) of the PTSD-susceptible B6N strain.

The decrease in pGSK-3β and β-catenin levels observed in the hippocampus of N/N vs. J/N and N/J vs. J/J (Fig. 7B) could not be observed in shocked vs. non-shocked B6N (Fig. 7A). Within the amygdala, however, the same changes in the AKT/GSK-3β/β-catenin pathway emerged in either case (Fig. 8A, B), suggesting it as the primary molecular signature of PTSD-like symptom severity. Interestingly, β-catenin was recently shown to play an important role in consolidation of fear memories at the level of the basolateral amygdala early after conditioning (Maguschak and Ressler, 2008). In the same study, data on β-catenin and remote fear memories were less conclusive, primarily because of a general temporal decay of the freezing responses in the animals (Maguschak and Ressler, 2008). Therefore, we can currently only speculate about the role of β-catenin in long-term maintenance of PTSD-like symptoms, in particular because no inhibitors of β-catenin are available and mutants differ in their genetic background from the PTSD-susceptible B6N strain (Maguschak and Ressler, 2008). It is conceivable that both transcriptional regulation of *Bdnf* expression (Wada, 2009) and direct interaction with the cytoskeleton (Salinas and Price, 2005) contribute to the morphological/functional changes of principal neurons observed after severe stress. Namely, hypertrophy within the basolateral amygdala and hypotrophy within the dorsal hippocampus (Vyas et al., 2002) may contribute to the development and/or maintenance of pathological anxiety in the aftermath of a traumatic/stressful experience.

As mentioned in the introduction, the stability of β-catenin depends on the activity status of GSK-3β. Under basal conditions, GSK-3β is dephosphorylated and, thus, constitutively active. Active GSK-3β destabilizes β-catenin via phosphorylation, which leads to proteasomal degradation of the transcription factor (Gould et al., 2008; Wada, 2009). GSK-3β, in turn, is inactivated by phosphorylation at the Ser 9 residue primarily through phosphorylated AKT. Inactivation of GSK-3β stabilizes β-catenin, which can then migrate into the nucleus and mediate gene transcription. Interestingly, GSK-3β seems to be hyperactive in schizophrenia and bipolar disorder. Also, a variety of antidepressants, antipsychotics and mood stabilizers may exert their therapeutic effects in part by inactivating GSK-3β (Beaulieu et al., 2009). Our findings add a new level of complexity to the potential role of the AKT/GSK-3β/β-catenin pathway in psychiatric disorders by demonstrating that (i) decreased rather than increased GSK-3β activity within the amygdala coincides with sustained PTSD-like symptoms in mice, and (ii) the same pathway appears to be regulated in the opposite manner in the amygdala and hippocampus.

CONCLUSION

In summary, our data demonstrate an important contribution of genetic factors to the individual susceptibility for developing PTSD-like symptoms at both the behavioural and molecular level. The changes in kinase activities observed in the PTSD-susceptible strain point to an active maintenance of the disease-like state months after the traumatic incident. In terms of human patients, the changes in the AKT/GSK-3β/β-catenin cascade may urge for the development of small chemical molecules that interfere with β-catenin signaling. This could hold promise of the development of novels drugs for the treatment of PTSD.

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REFERENCES

Afifi TO, Asmundson GJ, Taylor S, Jang KL (2010) The role of genes and environment on trauma exposure and posttraumatic stress disorder symptoms: a review of twin studies. Clin Psychol Rev 30:101–112.

Ahi J, Radulovic J, Spiess J (2004) The role of hippocampal signaling cascades in consolidation of fear memory. Behav Brain Res 149:17–31.

Balogh SA, Radcliffe RA, Logue SF, Wehner JM (2002) Contextual and cued fear conditioning in C57BL/6J and DBA/2J mice: context discrimination and the effects of retention interval. Behav Neurosci 116:947–957.

Beaulieu JM, Gainetdinov RR, Caron MG (2009) Akt/GSK-3 signaling in the action of psychotropic drugs. Annu Rev Pharmacol Toxicol 49:327–347.

Betancourt TS, Khan KT (2008) The mental health of children affected by armed conflict: protective processes and pathways to resilience. Int Rev Psychiatry 20:317–328.

Bremner JD, Elzinga B, Schmahl C, Vermetten E (2008) Structural and functional plasticity of the human brain in posttraumatic stress disorder. Prog Brain Res 167:171–186.

Copeland WE, Keeler G, Angold A, Costello EJ (2007) Traumatic events and posttraumatic stress in childhood. Arch Gen Psychiatry 64:577–584.

Cui Z, Wang H, Tan Y, Zaia KA, Zhang S, Tsien JZ (2004) Inducible and reversible NR1 knockout reveals crucial role of the NMDA receptor in preserving remote memories in the brain. Neuron 41:781–793.

Di Benedetto B, Kallnik M, Weisenhorn DM, Falls WA, Wurst W, Holter SM (2009) Activation of ERK/MAPK in the lateral amygdala of the

mouse is required for acquisition of a fear-potentiated startle response. Neuropsychopharmacology 34:356–366.
Foa EB, Stein DJ, McFarlane AC (2006) Symptomatology and psychopathology of mental health problems after disaster. J Clin Psychiatry 67(Suppl 2):15–25.
Francis DD, Szegda K, Campbell G, Martin WD, Insel TR (2003) Epigenetic sources of behavioral differences in mice. Nat Neurosci 6:445–446.
Frankland PW, O'Brien C, Ohno M, Kirkwood A, Silva AJ (2001) Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. Nature 411:309–313.
Galea S, Acierno R, Ruggiero K, Resnick H, Tracy M, Kilpatrick D (2006) Social context and the psychobiology of posttraumatic stress. Ann N Y Acad Sci 1071:231–241.
Gleason G, Liu B, Bruening S, Zupan B, Auerbach A, Mark W, Oh JE, Gal-Toth J, Lee F, Toth M (2010) The serotonin1A receptor gene as a genetic and prenatal maternal environmental factor in anxiety. Proc Natl Acad Sci U S A 107:7592–7597.
Gould TD, O'Donnell KC, Picchini AM, Dow ER, Chen G, Manji HK (2008) Generation and behavioral characterization of beta-catenin forebrain-specific conditional knock-out mice. Behav Brain Res 189:117–125.
Huang YY, Kandel ER (2007) 5-Hydroxytryptamine induces a protein kinase A/mitogen-activated protein kinase-mediated and macromolecular synthesis-dependent late phase of long-term potentiation in the amygdala. J Neurosci 27:3111–3119.
Kamprath K, Wotjak CT (2004) Nonassociative learning processes determine expression and extinction of conditioned fear in mice. Learn Mem 11:770–786.
Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB (1995) Posttraumatic stress disorder in the National Comorbidity Survey. Arch Gen Psychiatry 52:1048–1060.
Kozlovsky N, Matar MA, Kaplan Z, Kotler M, Zohar J, Cohen H (2007) Long-term down-regulation of BDNF mRNA in rat hippocampal CA1 subregion correlates with PTSD-like behavioural stress response. Int J Neuropsychopharmacol 10:741–758.
Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Tannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK, Nestler EJ (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131:391–404.
Maguschak KA, Ressler KJ (2008) Beta-catenin is required for memory consolidation. Nat Neurosci 11:1319–1326.
Millstein RA, Holmes A (2007) Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neurosci Biobehav Rev 31:3–17.
Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003) Manipulating the mouse embryo. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Paul S, Olausson P, Venkitaramani DV, Ruchkina I, Moran TD, Tronson N, Mills E, Hakim S, Salter MW, Taylor JR, Lombroso PJ (2007) The striatal-enriched protein tyrosine phosphatase gates long-term potentiation and fear memory in the lateral amygdala. Biol Psychiatry 61:1049–1061.
Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates. San Diego, CA: Academic Press.
Rose C, Rohl FW, Schwegler H, Hanke J, Yilmazer-Hanke DM (2006) Maternal and genetic effects on anxiety-related behavior of C3H/HeN, DBA/2J and NMRI mice in a motility-box following blastocyst transfer. Behav Genet 36:745–762.
Salinas PC, Price SR (2005) Cadherins and catenins in synapse development. Curr Opin Neurobiol 15:73–80.
Shan Q, Chan GC, Storm DR (2008) Type 1 adenylyl cyclase is essential for maintenance of remote contextual fear memory. J Neurosci 28:12864–12867.
Siegmund A, Dahlhoff M, Habersetzer U, Mederer A, Wolf E, Holsboer F, Wotjak CT (2009a) Maternal inexperience as a risk factor of innate fear and PTSD-like symptoms in mice. J Psychiatr Res 43:1156–1165.
Siegmund A, Kaltwasser SF, Holsboer F, Czisch M, Wotjak CT (2009b) Hippocampal N-acetylaspartate levels before trauma predict the development of long-lasting posttraumatic stress disorder-like symptoms in mice. Biol Psychiatry 65:258–262.
Siegmund A, Wotjak CT (2006) Toward an animal model of posttraumatic stress disorder. Ann N Y Acad Sci 1071:324–334.
Siegmund A, Wotjak CT (2007a) A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. J Psychiatr Res 41:848–860.
Siegmund A, Wotjak CT (2007b) Hyperarousal does not depend on trauma-related contextual memory in an animal model of posttraumatic stress disorder. Physiol Behav 90:103–107.
Suchecki D, Palermo NJ (1991) Prenatal stress and emotional response of adult offspring. Physiol Behav 49:423–426.
Trifilieff P, Calandreau L, Herry C, Mons N, Micheau J (2007) Biphasic ERK1/2 activation in both the hippocampus and amygdala may reveal a system consolidation of contextual fear memory. Neurobiol Learn Mem 88:424–434.
Trifilieff P, Herry C, Vanhoutte P, Caboche J, Desmedt A, Riedel G, Mons N, Micheau J (2006) Foreground contextual fear memory consolidation requires two independent phases of hippocampal ERK/CREB activation. Learn Mem 13:349–358.
Van den Hove DL, Blanco CE, Aendekerk B, Desbonnet L, Bruschettini M, Steinbusch HP, Prickaerts J, Steinbusch HW (2005) Prenatal restraint stress and long-term affective consequences. Dev Neurosci 27:313–320.
Van den Hove DL, Lauder JM, Scheepens A, Prickaerts J, Blanco CE, Steinbusch HW (2006) Prenatal stress in the rat alters 5-HT1A receptor binding in the ventral hippocampus. Brain Res 1090:29–34.
Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 22:6810–6818.
Wada A (2009) Lithium and neuropsychiatric therapeutics: neuroplasticity via glycogen synthase kinase-3beta, beta-catenin, and neurotrophin cascades. J Pharmacol Sci 110:14–28.
Wiltgen BJ, Silva AJ (2007) Memory for context becomes less specific with time. Learn Mem 14:313–317.
Yehuda R, Antelman SM (1993) Criteria for rationally evaluating animal models of posttraumatic stress disorder. Biol Psychiatry 33:479–486.

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