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Memory-updating abrogates extinction of learned immunosuppression

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ABSTRACT

When memories are recalled, they enter a transient labile phase in which they can be impaired or enhanced followed by a new stabilization process termed reconsolidation. It is unknown, however, whether reconsolidation is restricted to neurocognitive processes such as fear memories or can be extended to peripheral physiological functions as well. Here, we show in a paradigm of behaviorally conditioned taste aversion in rats memory-updating in learned immunosuppression. The administration of sub-therapeutic doses of the immunosuppressant cyclosporin A together with the conditioned stimulus (CS/saccharin) during retrieval blocked extinction of conditioned taste aversion and learned suppression of T cell cytokine (interleukin-2; interferon-γ) production. This conditioned immunosuppression is of clinical relevance since it significantly prolonged the survival time of heterotopically transplanted heart allografts in rats. Collectively, these findings demonstrate that memories can be updated on both neural and behavioral levels as well as on the level of peripheral physiological systems such as immune functioning.

1. Introduction

The ability to form, store and retrieve memories is an essential requisite of normal adaptive functioning for all organisms. When memories are recalled, they enter a transient labile phase in which they can be impaired or enhanced followed by a new stabilization process termed reconsolidation (Nader et al., 2000). Reconsolidation has been demonstrated for explicit and implicit, appetitive and aversive memories in various species including humans (Dudai, 2012; Schwabe et al., 2014). It is unknown, however, whether reconsolidation of behavioral conditioned physiological responses such as immunological functions is possible. Bidirectional interaction between the brain and peripheral immune system (Dantzer et al., 2008; Tracey, 2010), together with the fact that immune functions can be modified by associative learning processes (Schedlowski and Pacheco-Lopez, 2010) provide a unique opportunity to test this assumption.

Behavioral conditioning of immune responses has been demonstrated in both rodents and humans (Schedlowski and Pacheco-Lopez, 2010). In our established conditioned taste aversion model, rats are presented with a novel taste (saccharin solution; conditioned stimulus, CS) and are subsequently injected with the immunosuppressive drug cyclosporin A (CsA; unconditioned stimulus, US) three times during acquisition (Fig. 1A). CsA is a clinically used selective calcineurin inhibitor that suppresses the production of cytokines, in particular interleukin (IL)-2 and interferon (IFN)-γ, by activated T lymphocytes (Kahan, 1989; McCaffrey et al., 1993). During retrieval, animals are re-exposed three times to the CS, inducing behaviorally conditioned taste aversion (CTA) and more importantly, significantly diminished *ex vivo* IL-2 and IFN-γ production by anti-CD3 stimulated splenic T cells (Exton et al., 2002, 1998; Pacheco-Lopez et al., 2005).

As with every other learning process, however, behaviorally conditioned immunosuppression is subject to extinction. The potential clinical relevance of this learned immunosuppression has therefore often been questioned since a progressive decrease in the learned immunosuppressive response over time is a considerable problem for the systematic application of conditioning paradigms as a supportive treatment option in immunopharmacological regimens (Doering and Rief, 2012; Hadamitzky et al., 2013).

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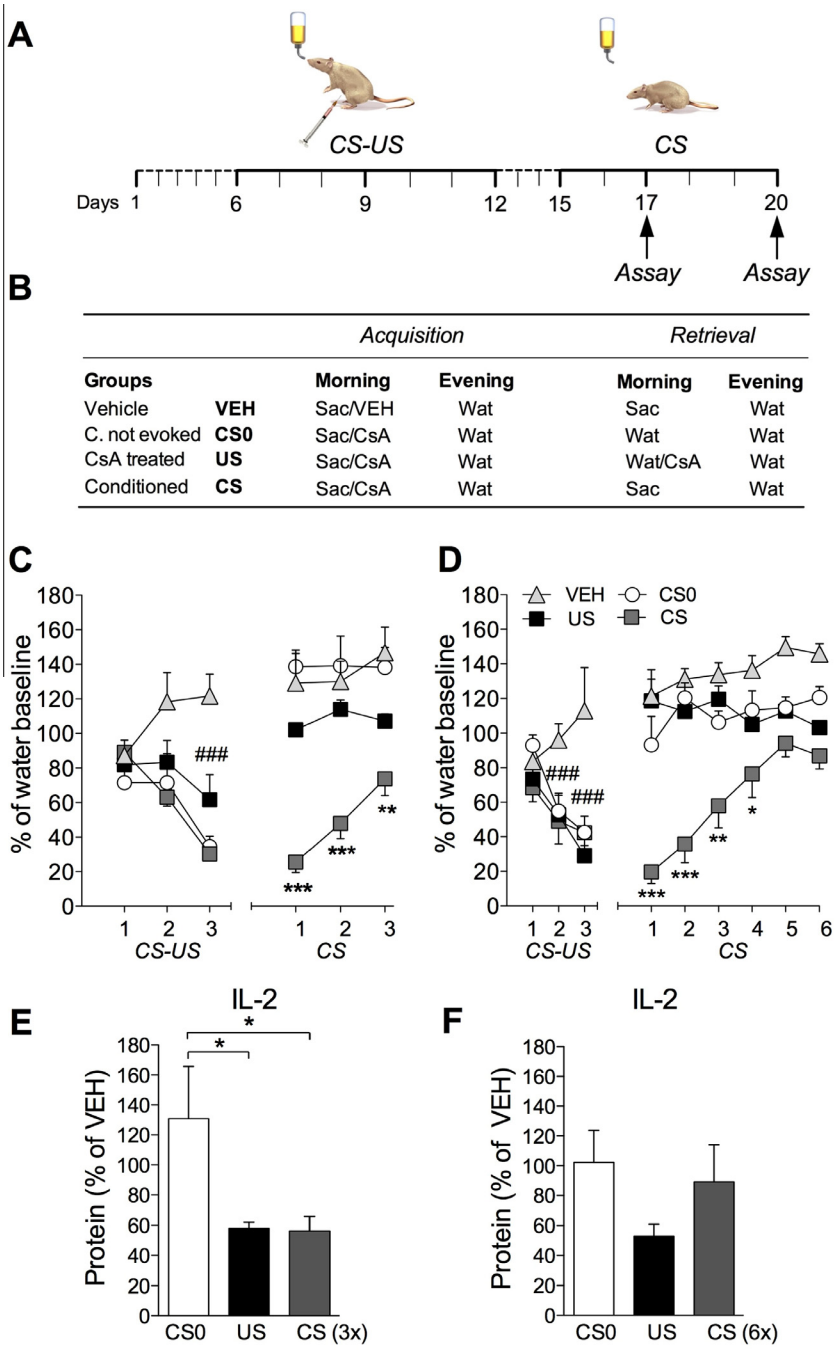


Fig. 1. Behavioral task, group allocation and extinction pattern of behaviorally conditioned immunosuppression. (A) Schematic representation of the experimental protocol. For acquisition, rats received three CS–US (saccharin (Sac)-CsA) training trials and were re-exposed to the CS during retrieval either three or six times. (B) Group allocation and treatment design. Animals in the conditioned (*CS*), conditioned not-evoked (*CS0*), as well as in the pharmacological control group (*US*) were conditioned either with Sac and CsA (20 mg/kg, i.p.) during acquisition; rats in the control group (*VEH*) received an injection of vehicle together with Sac. During retrieval, animals in the CS group were re-exposed to Sac, the US groups received water and three or six CsA (20 mg/kg, i.p.) injections. *CS0* animals were re-exposed to water only, VEH animals received sac only. (C and D) Taste aversion index in behavioral conditioning with three and six retrieval trials. During the first acquisition trial, animals in all groups showed a neophobic response to the CS. The *US*-, *CS0*-, and *CS*-groups displayed pronounced taste avoidance during the second and/or third CS re-exposures ($^{###}P < 0.001$, decrease in fluid consumption of all conditioned groups relative to *VEH*, ANOVA followed by Bonferroni's test). Two days later, rats were re-exposed to the taste stimulus for three or six consecutive retrieval trials. (C) After three CS re-exposures rats in the *CS*-group exhibited pronounced avoidance towards the taste stimulus, whereas after (D) six CS re-exposures taste avoidance was extinguished in the CS-group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, decreased fluid consumption relative to CS0, ANOVA followed by Bonferroni's test). (E and F) Contents of IL-2 cytokine levels measured by ELISA. The CS-group displayed marked conditioned suppression of IL-2 production after (E) three but not after (F) six CS re-exposures ($^{*}P < 0.01$, ANOVA followed by Bonferroni's test). Asterisks and crosses represent a statistically significant difference between indicated groups (*VEH*, $n = 9$; *US*, $n = 8–9$; *CS0*, $n = 6–9$; *CS*, $n = 7–9$). Data are means ± SEM.

Research on the extinction of fear memory focuses on the question as to how the extinction process can be accelerated and reconsolidation of acquired memory blocked. In contrast, in the context of behaviorally conditioned immune responses, the major issue is how extinction of the learned immunosuppression can be prevented in order to preserve the conditioned immune response. Consolidated memories can re-enter states of transient instability following reactivation called the 'reconsolidation window', during

which memories can either be re-established or blocked by amnestic agents, but remain unaffected outside this transient phase of instability (de Carvalho Myskiw et al., 2014; Eisenberg et al., 2003; Garcia-DeLaTorre et al., 2009; Monfils et al., 2009; Schiller et al., 2010). Previous studies employing a CTA paradigm with lithium chloride as US demonstrated that a very low dose of the US is unable to induce CTA *per se* and administered together with the CS during retrieval, diminished CTA extinction and re-established the original taste aversion score, indicating a reconsolidation process (Berman et al., 2003). Against this background, here we aimed at analyzing possible reconsolidation processes in peripheral immune responses. Thus, we first investigated the extinction of a behaviorally conditioned immunosuppressive response, and second, whether extinction of this learned immunosuppression could be abrogated by applying sub-therapeutic doses of the US (i.e., CsA) in combination with the CS during retrieval. Third, we analyzed the potential clinical relevance of blocked extinction of learned immunosuppressive responses.

2. Methods

2.1. Animals

Male Dark Agouti rats (DA/HanRj, 200–230 g; Janvier, France) were single housed with *ad libitum* access to food. Tap water was available *ad libitum* until the water deprivation regimen started. The vivarium was temperature (20 °C) and humidity (55 ± 5%) controlled and maintained on a reversed 12/12-h light/dark cycle (7:00 a.m. to 7:00 p.m.), to enable the experiments to be conducted during the activity phase of the rats' awake/sleep cycle. Animals were allowed to acclimate to their new surroundings for 2 weeks before initiation of any experimental procedure. The animal facilities and experimental procedures were in accordance with National Institutes of Health and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee (LANUV Düsseldorf, North Rhine-Westphalia). For the transplantation experiments, DA recipients and Lewis (LEW) (Lewis, donor, Janvier, France) strains were used at 200–230 g of weight.

2.2. Behavioral conditioning protocol

As in previous studies, a stock solution (100 mg/ml) of cyclosporin A (CsA; LC Laboratories, Woburn, USA) dissolved in 100 μL ethanol (96%), and 900 μL Miglyol (Caelo, Germany) was further diluted with sterile saline (0.9% NaCl, Braun, Germany) to adjust for the required drug dose of 20 mg/kg body weight at a final injection volume of 1 ml administered intraperitoneally (i.p.) (Hadamitzky et al., 2015). The current conditioning paradigm employed two drinking sessions per day. Specifically, rats were subjected to a water deprivation regime for 5 days, allowing them to drink for 15 min at 8:45 a.m. and again at 5 p.m. each day. During the morning session on the fifth day, the acquisition phase started and a drinking solution containing 0.2% (w/v) sodium saccharin (Sigma–Aldrich, Schnelldorf, Germany) as conditioned stimulus (CS) was paired with an injection of 20 mg/kg CsA as the unconditioned stimulus (US) in conditioned (*CS*), CsA-treated (*US*) as well as conditioned-not evoked (*CS0*) animals, whereas vehicle animals (*VEH*) received saccharin together with a saline injection (Fig. 1A and B). This protocol was repeated three times with CS–US learning trials separated by 72 h. All animal groups received water during the evening session of the three acquisition trials. Two days following the last acquisition trial, three or six retrieval trials, respectively, were performed separated by 24 h intervals. During the retrieval trials, conditioned rats (*CS*) received saccharin in the morning and water in the afternoon sessions. The *US*-group received water in the morning and evening sessions and three or six injections with CsA (20 mg/kg) respectively, serving as the pharmacological control. The *CS0*-group received water during both morning and evening sessions. Finally, a vehicle (*VEH*) control group was treated exactly like the *CS*-group, although they were injected with sterile saline (0.9% NaCl; Braun Medical, Germany) rather than CsA during acquisition trials. Before and after each drinking session, the drinking bottles were weighed to measure fluid consumption. One hour after the third or sixth CS re-exposure, respectively, animals were sacrificed for immunological analyses.

2.3. Behavioral conditioning in the transplantation experiments

Dark Agouti recipient animals were randomly allocated to one of four treatment groups (Fig. 4B). Animals in the *Rec*-, *Nrec*-, and *CS0*-groups all received saccharin as the CS and CsA as the US during the three acquisition trials (see above). Control animals served as the home cage control group. Daily fluid intake of all conditioned groups (*Rec*, *Nrec*, *CS0*) was restricted to two 15 min drinking sessions (water or saccharin, respectively, during morning and evening drinking sessions) throughout the entire experiment. During the retrieval (2 days after the last acquisition trial's morning drinking session), animals were presented with either a combination of CS and a sub-therapeutic dose of CsA (*Rec*-group, reminder cue within the reconsolidation window), the CS alone (*Nrec*-group), or water (*CS0*-group) on three subsequent days until heart transplantation. During the evening drinking sessions 8 h later, the *Nrec*-group animals received water together with an injection of sub-therapeutic CsA (2 mg/kg) (outside the reconsolidation window). The *CS0*-group animals received just water. Heterotopic heart transplantations were performed immediately after the third retrieval trial. Retrieval was then continued for another 10 days. If the allograft had not been rejected during those days, the animals were observed until graft rejection with *ad libitum* access to water.

2.4. Sub-therapeutic dose-finding experiment

To determine sub-therapeutic CsA, animals were injected with different doses of CsA (20 mg/kg, 10 mg/kg, 5 mg/kg, and 2 mg/kg) on six consecutive days. On the last day, 1 h after the injection, animals were sacrificed and immunologically analyzed. The CTA dose–response experiment essentially followed the experimental design described in the behavioral-conditioning protocol except for the CsA dose employed. After 5 days of water deprivation, animals received three saccharin-CsA (CS-US) pairings (5 and 2 mg/kg) for acquisition and were then re-exposed to the CS for six consecutive days. A vehicle-treated control group (*VEH*) received saccharin and was injected with saline on acquisition days. Fluid consumption was assessed to monitor conditioned taste aversion.

2.5. CsA quantification

To determine the amount and distribution of the sub-therapeutic CsA dose in blood and brain samples, two sets of animals were injected with either 2 mg/kg or 20 mg/kg CsA over six consecutive days and sacrificed by decapitation 120 min and 240 min following the last CsA injection. Trunk blood was collected in EDTA-treated tubes (Monovette, Sarstedt, Nümbrecht, Germany). Plasma was separated by centrifugation (2000*g*, 10 min, 4 °C) and stored at −80 °C until further analysis. Brains were quickly removed, dissected into cerebellum and cerebrum, frozen on dry ice, and stored at −80 °C. Using a freezing microtome (Microm HM560, Thermo Fisher Scientific, Walldorf, Germany), coronal brain sections 200 μm thick were cut at −5 °C and placed

on pre-chilled glass slides. The amygdala and insular cortex were then dissected from serial brain sections using a micropunch technique (Cuello and Carson, 1983; Hadamitzky et al., 2014). Briefly, a pre-chilled stainless steel sample puncher (internal diameter of 2 mm; Fine Science Tools, Heidelberg, Germany) was used to obtain tissue samples of the left and right insular cortex and amygdala. Optical tract and hippocampus served as anatomical landmarks to ensure comparable positions of the punched samples across animal (Paxinos and Watson, 1998). CsA concentrations in peripheral blood, spleen tissue and brain tissue were analyzed as described previously (Christians et al., 2004; Gottschalk et al., 2011). Briefly, samples were thawed, weighed and homogenized with 1 M KH_2PO_4 buffer (pH = 7.4). After protein precipitation with methanol/$ZnSO_4$ 0.4 M (80:20, v/v), samples were vortexed for 30 s and centrifuged (15,000×*g*, 3 min). 100 µL of the supernatant were injected into a HPLC-MS/MS system and analyzed in reference to CsA.

2.6. Memory-updating of CTA and learned immunosuppression

The conditioning protocol basically followed the conditioning protocol with six CS re-exposures described above and identically treated *US*-, *CS0*-, and *VEH*-groups. However, two additional experimental groups (*Rec* and *Nrec*) were now included in the protocol. Both groups were conditioned with three acquisition trials receiving saccharin as CS and an i.p. injection with CsA (20 mg/kg) as US as described above. During each of the six retrieval trials however, the *Rec*-group animals received an i.p. injection with subtherapeutic CsA (2 mg/kg) immediately after saccharin exposure in the morning session (within the reconsolidation window) and water together with a vehicle injection in the evening session. In contrast, the *Nrec*-group received saccharin together with a vehicle i.p. injection in the morning session and water together with CsA administration (2 mg/kg) outside the reconsolidation window (8 h after the CS/saccharin administration) during the evening drinking session. One hour after the sixth morning drinking session, animals were sacrificed and immunological analyses performed. Data are pooled from 3 independent experiments and data shown as percentage changes from vehicle controls.

2.7. Splenocyte isolation and stimulation

Single-cell suspensions of the spleen were obtained by mechanically disrupting the tissue with a syringe plunger in cold Hanks' balanced salt solution (Invitrogen, Basel, Switzerland). Red blood cells were removed using diluted PharM Lyse (BD Pharmingen, Allschwil, Switzerland). Splenocytes were washed in cell culture medium (RPMI 1640 supplemented with GlutaMAX I, 25 mM HEPES, 10% FBS, and 50 µg/ml gentamicin; Invitrogen) and filtered through a 70 µm nylon cell strainer. Cell concentrations were determined with an automatic animal cell counter (Vet abc; Medical Solution, Steinhausen, Switzerland), and splenocytes adjusted to a final concentration of 5×10^6 cells/mL. Splenocytes were stimulated in 96-well flat-bottom microtiter plates with 1 µg/ml of mouse anti-rat CD3 monoclonal antibody (clone: G4.18; BD Pharmingen, Heidelberg, Germany) for 48 h in a humidified incubator (37 °C, 5% CO_2).

2.8. Cytokine analyses

Culture supernatants of non-stimulated and anti-CD3-stimulated splenocytes were collected after 48 h of incubation, and IL-2 and IFN-γ concentrations were measured by ELISA (IL-2 ELISA, Biosource; IFN-γ ELISA Invitrogen) according to the manufacturer's instructions.

2.9. Gene expression analysis

Splenocytes (2.5×10^6) were stimulated with 1 µg/mL of mouse anti-rat CD3 monoclonal antibody (clone: G4.18; BD Pharmingen) for 4 h in a humidified incubator (37 °C, 5% CO_2). After incubation, cells were washed with phosphate-buffered saline (PBS; Invitrogen) and total RNA was extracted using the RNeasy Mini Kit and RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Single-strand cDNA was generated by reverse transcription using MultiScribe reverse transcriptase and random hexamer primers (Applied Biosystems, Darmstadt, Germany). The mRNA expression levels of the target genes were determined by real-time PCR using Taqman gene expression assays (Applied Biosystems) for IL-2 (Rn00587673_m1) and IFN-γ (RN00594078_m1) with 18s RNA (#4319413E) as endogenous control gene. Amplification and detection were done on a 7500 FAST Real-Time PCR system (Applied Biosystems). Data are pooled from 3 independent experiments and shown as percentage changes from vehicle controls.

2.10. Heterotopic heart transplantation

The heterotopic heart transplantation from Lewis to Dark Agouti rats was performed as described before (Ono and Lindsey, 1969) with slight modifications (Wang et al., 2007). Rats were anesthetized in an induction chamber with 5% isoflurane and 2% oxygen. Anesthesia was maintained by a flow of 1.5–2% isoflurane and oxygen (1 l/min) through a mask during surgery. After explantation, the donor heart was perfused with NaCl and 1000 I.E. heparin. The graft was then placed into the recipient's abdominal cavity where the abdominal vessels had already been prepared (preparation and longitudinal incision in the recipient's vena cava and abdominal aorta). The heterotopic transplantation was conducted by end-to-side anastomosis of the donors ascending aorta to the recipient abdominal aorta, and of the donor pulmonary trunk to the recipient inferior vena cava. Graft function was assessed via abdominal palpation (Martins, 2008) once daily by two investigators blind to that animal's treatment. Rejection was defined as the absence of a palpable graft contraction, which was confirmed by visual affirmation after laparotomy.

2.11. Statistical analyses

Experimental numbers of animals are reported in the figure legends and method section. Experiments in Fig. 3 were repeated three times with at least $n = 5$ animals in each of the experimental groups in each experiment. All statistical analyses were performed using SigmaPlot software (Version 12.3, SPSS, Chicago, IL, USA) and results are calculated as means with error bars representing the SEM. Analyses were performed using ANOVAs when assessing three-group comparisons (cytokines, mRNA), and by means of ANOVAs with within-subjects design for multiple group comparisons (CTA). Separate ANOVAs were calculated for the three acquisition trials and the three or six evocation trials, respectively. When appropriate, post hoc individual comparisons between groups were determined by Bonferroni's corrections; the level of significance was set at $P < 0.05$. For non-parametric testing (subtherapeutic dose finding, CsA quantification) the Mann–Whitney-*U* test was used.

3. Results

3.1. Analyzing extinction at the behavioral and immune system levels

Animals received three conditioning trials during acquisition and were re-exposed to the CS during retrieval on either three or six subsequent days (Fig. 1A and B). Three re-exposures to the CS induced a significant CTA (Fig. 1C) (ANOVA; $F(3, 56) = 11.696$, $P < 0.001$) as well as a significant behaviorally conditioned reduction in the IL-2 protein production by anti-CD3 stimulated splenic T cells (Fig. 1E) (ANOVA; $F(2, 18) = 5.131$, $P < 0.017$). In contrast, following six subsequent CS re-exposures both the CTA (Fig. 1D) (ANOVA; $F(3, 155) = 23.302$, $P < 0.001$) and the learned suppression of IL-2 production (Fig. 1F) (ANOVA; $F(2, 18) = 1.704$, $P = 0.210$) were completely extinguished.

3.2. Determine sub-therapeutic doses of CsA

To identify doses of CsA below therapeutic level, we first analyzed the effects of six consecutive daily injections of 2, 5, 10, or 20 mg/kg CsA on *ex vivo* IL-2 and IFN-γ production by anti-CD3 stimulated splenic T cells. We observed a significant suppressive effect on both cytokines in animals treated with 20 mg/kg ($P < 0.01$; $P < 0.05$), 10 mg/kg ($P < 0.05$; $P < 0.05$), and 5 mg/kg ($P < 0.01$; $P < 0.05$) of CsA, respectively, compared to vehicle. In contrast, 2 mg/kg CsA did not significantly affect IL-2 ($P = 0.239$) and IFN-γ ($P = 0.751$) production (Fig. 2A and B). We subsequently tested the effect of the two lowest CsA doses on the CTA response during acquisition and retrieval. A dose of 5 mg/kg CsA remained capable of inducing moderate CTA during acquisition (third CS; $P < 0.05$) and retrieval (CS re-exposure 1, 2, 3, 5; $P < 0.05$), whereas administration of 2 mg/kg CsA did not induce a CTA (Fig. 2C). Finally, we analyzed CsA concentrations in peripheral blood, spleen tissue, and in brain areas known to mediate the CsA-induced CTA and learned immunosuppression such as the insular cortex and the amygdala (Pacheco-Lopez et al., 2005). Six consecutive daily i.p. injections of 20 mg/kg CsA significantly increased CsA concentrations 120 and 240 min after administration in peripheral blood (120 min, $P < 0.001$; 240 min, $P < 0.01$), and spleen tissue ($P < 0.01$ for both time points), as well as in the insular cortex ($P < 0.01$ for both time points), and the amygdala (120 min, $P < 0.01$, 240 min, $P < 0.05$). In contrast, CsA levels were detectable neither in the periphery nor in the different brain areas 2 and 4 h after administration of 2 mg/kg CsA (Fig. 2D–G).

3.3. Memory-updating blocks extinction of learned immunosuppression

Previous findings demonstrated a re-consolidation effect in CTA after administration of sub-effective LiCl doses in combination with the CS (Berman et al., 2003). Thus, in the next step, we conducted an experiment in which we administered a sub-therapeutic CsA dose (2 mg/kg) during each retrieval trial either concomitantly with the CS (i.e., within the re-consolidation window) (*Rec*-group) or 8 h after CS re-exposure (i.e., outside the re-consolidation window) (*Nrec*-group). ANOVA showed a significant group effect for CTA during retrieval ($F(4, 390) = 50.047$, $P < 0.001$). Post-hoc analyses revealed that in the *Rec*-group CTA extinction was significantly

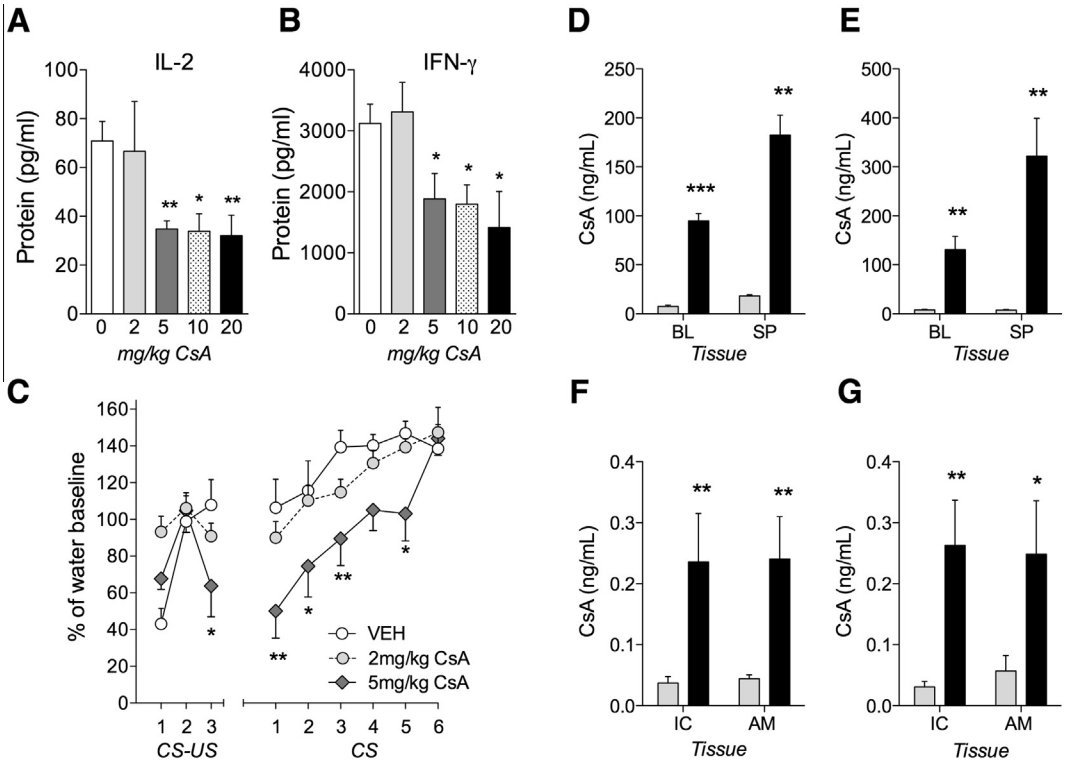


Fig. 2. Sub-therapeutic CsA dose finding. (A) Contents of IL-2 and (B) IFN-γ measured by using ELISA. Six consecutive daily injections with CsA induced significant suppression of cytokine production after 5, 10, and 20 mg/kg administration but not after 2 mg/kg ($^{*}P < 0.05$, and $^{**}P < 0.01$ relative to vehicle-treated controls, Mann–Whitney-*U* test; $n = 5$–6 per group). (C) Saccharin aversion index in rats behaviorally conditioned with 2 or 5 mg/kg CsA, compared to vehicle-treated controls. Only animals in the 5 mg/kg CsA group displayed conditioned taste aversion to saccharin during acquisition and retrieval, respectively ($^{*}P < 0.05$, and $^{**}P < 0.01$ relative to controls, ANOVA followed by Bonferroni's test; $n = 8$ per group). (D–G) Peripheral and central CsA levels. Six consecutive daily injections of 20 mg/kg but not with 2 mg/kg CsA significantly increased CsA concentrations 120 and 240 min after administration in (D and E) peripheral blood (BL) and spleen tissue (SP) as well as in (F and G) the insular cortex (IC) and the amygdala (AM) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Mann–Whitney-*U* test; $n = 5$ per group). Asterisks and crosses represent a statistically significant difference between indicated groups. Data are means ± SEM.

delayed compared to the *Nrec*-group (days 3–5, $P < 0.001$; day 6, $P < 0.05$) (Fig. 3A). In parallel with the inhibited CTA, ANOVA revealed a significant group effect in anti-CD3-stimulated cytokine production at protein (IL-2: $F(3, 55) = 8.209$, $P < 0.001$); IFN-γ: $F(3, 61) = 25.910$, $P < 0.001$) (Fig. 3B and C) and mRNA levels (IL-2: $F(3, 55) = 13.430$, $P < 0.001$), (IFN-γ: $F(3, 53) = 4.978$, $P = 0.004$) (Fig. 3D and E) after the 6th CS re-exposure. Post hoc analyses showed that cytokine protein (IL-2: $P = 0.046$; IFN-γ: $P < 0.001$) and mRNA expression (IL-2: $P = 0.012$; IFN-γ: $P < 0.013$) in the *Rec*-group were significantly diminished compared to animals receiving sub-therapeutic CsA treatment outside the reconsolidation window (*Nrec*-group), suggesting that reconsolidation-like processes are both evident on a behavioral level (CTA) and inducible in peripheral physiological systems such as immune functioning.

3.4. Memory-updating of learned immunosuppression is able to prolong survival time of heterotopically transplanted heart allografts

The calcineurin inhibitor CsA is widely used in transplantation medicine to prevent the rejection of transplanted organs (Halloran et al., 1999). Thus, we tested whether reconsolidation-like processes in learned immunosuppression would be capable of delaying the rejection of a transplanted vascularized organ. Therefore, conditioned Dark Agouti rats were transplanted with heterotopic heart allografts from histo-incompatible Lewis donor rats following the third CS representation (Fig. 4A). During retrieval, animals in the Rec- and Nrec-groups were administered sub-therapeutic doses of CsA (2 mg/kg) on the first three retrieval days and on 10 consecutive days thereafter as follows; the Rec-group animals received CsA immediately after each CS representation; the Nrec-group received CsA in the evening drinking session together with water (Fig. 4B). Mean heart allograft survival time was significantly prolonged in *Rec*-group's conditioned animals that had received the subtherapeutical reminder US (2 mg/kg CsA) together with the CS within the reconsolidation window (Fig. 4C) (*Rec* vs. *Nrec* $P = 0.013$, *Rec* vs. *CS0* $P = 0.001$, *Rec* vs. control, $P < 0.001$), suggesting that the reconsolidation-like processes of learned immunosuppression can be clinically relevant.

4. Discussion

Our results demonstrate that extinction of CTA and, more importantly, conditioned suppression of IL-2 and IFN-γ cytokine responses can be abrogated when administering sub-therapeutic doses of the immunosuppressant CsA as a reminder cue in parallel with the CS during retrieval. In contrast, sub-therapeutic CsA was completely ineffective when administered 8 h after CS re-exposure. These data suggests that the timing of the reminder cue during the labile phase of the memory trace following retrieval (i.e., inside vs. outside the reconsolidation window) is crucial for initiating the reconsolidation process involving de novo protein synthesis (Agren, 2014; Alberini and Ledoux, 2013; de Carvalho Myskiw et al., 2014; Schiller et al., 2010; Tronson and Taylor, 2007). Furthermore, this updated learned immunosuppressive response is of clinical relevance, since it significantly prolonged the survival time of heterotopically transplanted hearts.

First evidence from CTA experiments in rats demonstrated that the passage of time is crucial for initiating a reconsolidation process in CTA. Blocking protein synthesis in the insular cortex within 6 h after re-exposition to the CS (saccharin taste) during retrieval, but not outside this reconsolidation window, significantly impaired the extinction process (Eisenberg et al., 2003; Garcia-DeLaTorre et al., 2009). In addition, employing low doses of the US (lithium chloride) together with the CS during the extinction process, which was ineffective in inducing CTA in naive rats,

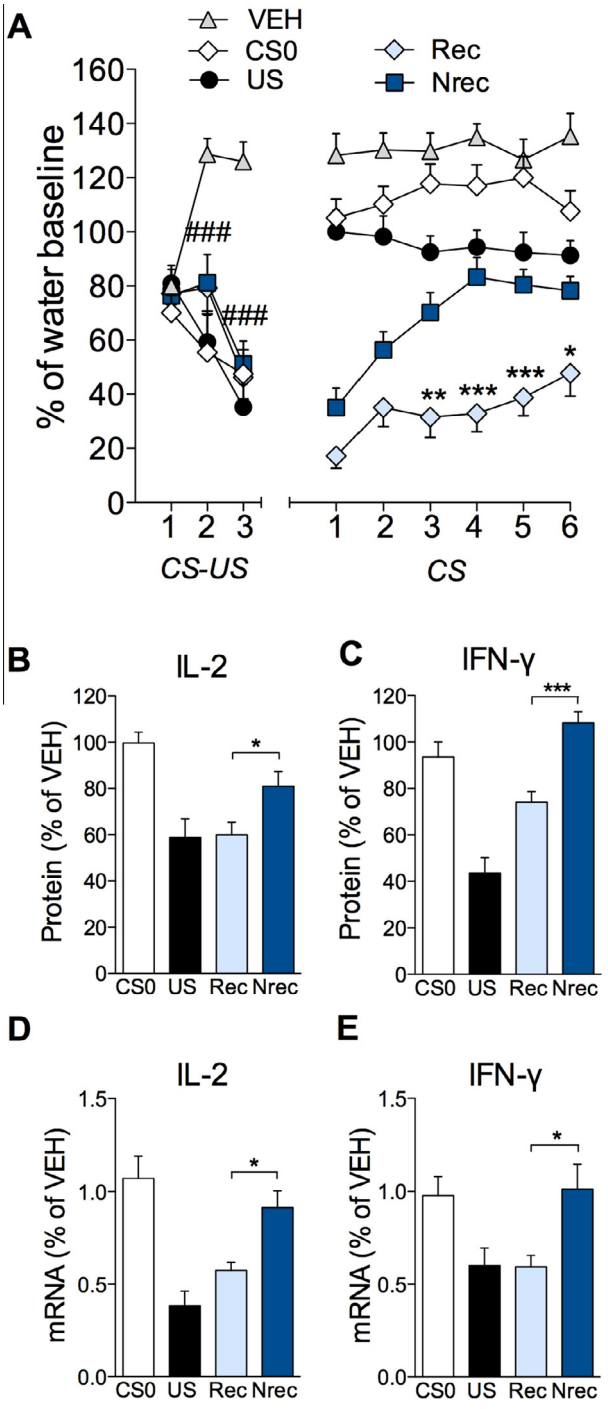
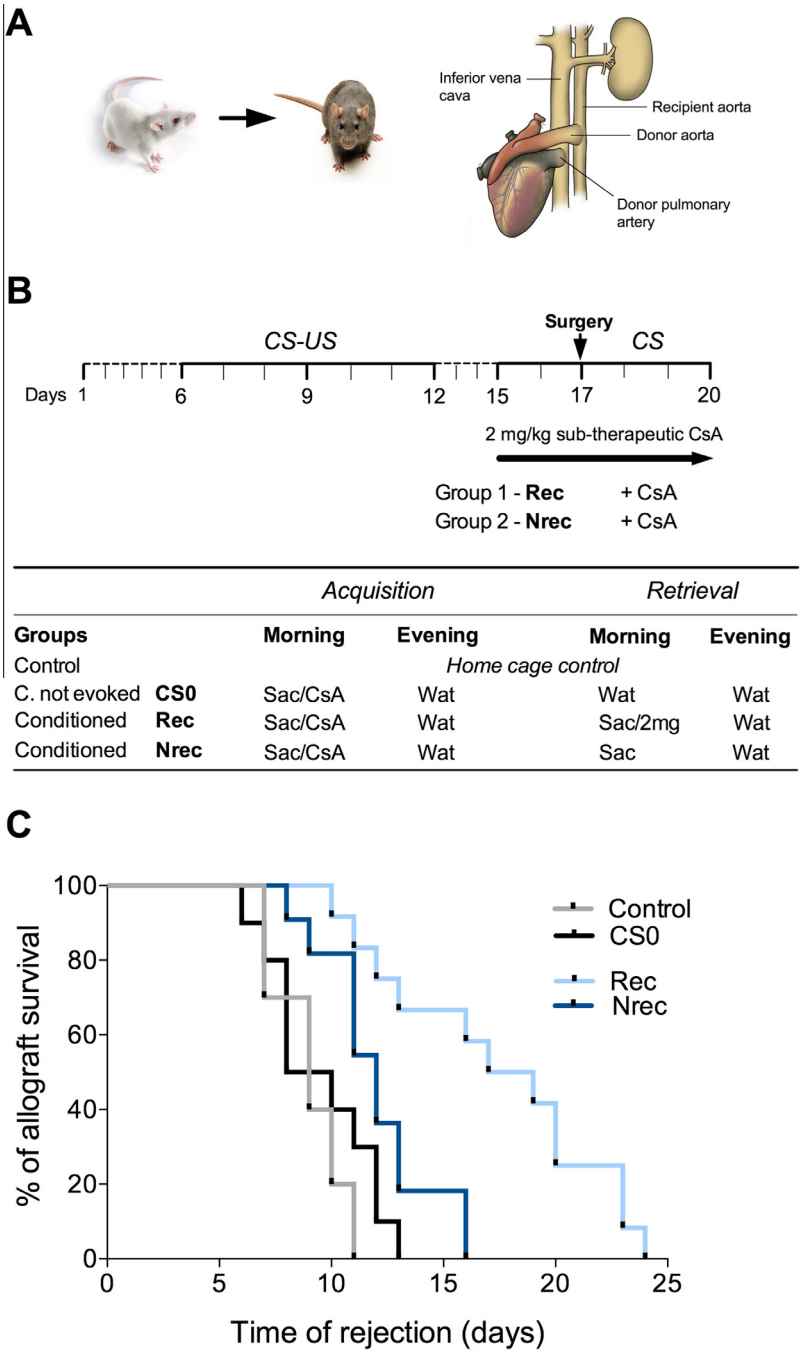


Fig. 3. Memory-updating of CTA and learned immunosuppression. (A) Saccharin aversion index. For acquisition, rats received three CS–US (saccharin (Sac)-CsA) training trials and all conditioned groups displayed CTA at the second and third acquisition trials ($^{\#\#\#}P < 0.001$, decrease in fluid consumption relative to *VEH*, Bonferroni's test). During retrieval, injections with sub-therapeutic CsA (2 mg/kg) together with Sac (within the reconsolidation window) diminished CTA extinction in the *Rec*-group compared to animals (*Nrec*-group) injected with the reminder cue 8 h after Sac re-exposures (outside the reconsolidation window) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, ANOVA followed by Bonferroni's test). (B–D) Protein release and mRNA expression of the cytokines IL-2 and IFN-γ measured by ELISA. Sub-therapeutic reminder cues given together with the CS significantly prevented the extinction of learned suppression in IL-2 protein release (B) and mRNA expression (D), as well as of IFN-γ protein production (C) and mRNA expression (E) in the *Rec*-group compared to the *Nrec*-group ($^{*}P < 0.05$, and $^{***}P < 0.001$, ANOVA followed by Bonferroni's test). Results are merged from three independent experiments and are shown as mean percentage changes from vehicle controls. Asterisks and crosses represent a statistically significant difference between indicated groups (*VEH*, $n = 17$; *US*, $n = 14$–16; *CS0*, $n = 11$–12; *Rec*, $n = 16$–18; *Nrec*, $n = 16$–19). Data are means ± SEM.



		Acquisition		Retrieval	
Groups		**Morning**	**Evening**	**Morning**	**Evening**
Control			*Home cage control*		
C. not evoked	**CS0**	Sac/CsA	Wat	Wat	Wat
Conditioned	**Rec**	Sac/CsA	Wat	Sac/2mg	Wat
Conditioned	**Nrec**	Sac/CsA	Wat	Sac	Wat

Fig. 4. Memory-updating of learned immunosuppression prolongs heart allograft survival. (A) Schematic representation of Dark Agouti (DA) rats that received a heterotopically transplanted donor heart from major histocompatibility complex (MHC)-incompatible Lewis (LEW) rats. (B) Group allocation and treatment design for conditioning. For acquisition, animals in the *Rec*-, *Nrec*-, and *CS0*-groups received three combination trials of saccharin (Sac) and an injection of 20 mg/kg CsA. During retrieval, injections with sub-therapeutic 2 mg/kg CsA together with Sac (within the reconsolidation window) were given to the *Rec*-group compared to animals (*Nrec*) injected with the reminder cue 8 h after Sac re-exposures (outside the reconsolidation window) for 13 consecutive days. The *CS0*-group received water throughout retrieval and control animals served as home cage controls and were not integrated in the conditioning protocol. (C) Kaplan–Meier survival curve of transplanted heart allografts. Injections with sub-therapeutic CsA (2 mg/kg) together with the CS within the reconsolidation window for 10 consecutive days during retrieval significantly prolonged the survival time of heart allografts (*Rec*-group) compared to animals receiving the 2 mg/kg CsA outside (*Nrec*-group) the reconsolidation window ($P = 0.013$, Mann–Whitney-*U* test; $n = 11–12$ per group). Data are means ± SEM.

regained the original aversion score, as the rats had never been subjected to the extinction procedure (Berman et al., 2003). In the model employed here, the behaviorally conditioned suppressive effects on IL-2 and IFN-γ cytokine production and other T cell functions were shown to be centrally mediated via the insular cortex and amygdala, and peripherally via sympathetic noradrenergic fibers and the adrenoceptor-dependent inhibition of calcineurin activity (Exton et al., 2002, 1999, 1998; Pacheco-Lopez et al., 2005, 2009; Riether et al., 2011). The data presented here suggest that neural processes mediating memory-updating are transferred to peripheral immune functions via these mechanisms (Schedlowski and Pacheco-Lopez, 2010).

The neural mechanisms of extinction learning and memory reconsolidation are not only analyzed for a better understanding

of memory processes in general. Research also focuses on the question as to how the extinction process can be accelerated and reconsolidation of acquired memory blocked in order to develop new strategies for the treatment of anxiety or posttraumatic stress disorders (Agren, 2014). In contrast, in the context of behaviorally conditioned immune response the ultimate aim is to abrogate or block extinction of learned immunosuppression in order to employ these learning paradigms in clinical situations as supportive therapy (Schedlowski and Pacheco-Lopez, 2010). The potential clinical relevance of learned immunosuppression has been repeatedly shown in rodents (Ader and Cohen, 1982; Exton et al., 1999; Schedlowski and Pacheco-Lopez, 2010). Here we demonstrate, that blocking the extinction of learned immunosuppression by implementing (sub-therapeutical) reminder cues in parallel with the CS significantly prolonged the rejection of a vascularized allograft. Employing a taste-CsA paradigm, behavioral conditioned immunosuppression could also be induced in humans (Goebel et al., 2002; Ober et al., 2012), showing recently that extinction of learned immunosuppression could be delayed by administering subtherapeutical reminder cues during CS-re-exposure (Albring et al., 2014). Together these data suggest that reconsolidation-like processes may serve as an adaptive update mechanism not only for motor, declarative, emotional memories or sensory processes (Bonin and De Koninck, 2014) but also for CNS-induced memories in peripheral physiological functions providing the unique opportunity to strengthen adaptive memories without requiring the original learning situation.

These data can be taken as a model to implement learning protocols as a supportive therapy in immunopharmacological regimens (Albring et al., 2014). Further analysis of the basic mechanisms of extinction learning and reconsolidation of learned immune responses will be essential though to better understand the brain-immune system communication in general. In particular to achieve the long term goal of the learned immune response: employing these learning paradigms in clinical situations as supportive therapy together with standard pharmacological regimen with the aim to maximize the therapeutic outcome for the patient's benefit (Doering and Rief, 2012; Enck et al., 2013; Schedlowski et al., 2015).

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