Through the eye to the brain: Modulating LC-mediated arousal via pupil-based neurofeedback

Sarah Nadine Meissner[a]*, Marc Bächinger[a], Sanne Kikkert[a], Jenny Imhof[a], Silvia Missura[a], Manuel Carro Dominguez[a,b] , Nicole Wenderoth[a,b,c]*

[a]Neural Control of Movement Laboratory, Department of Health Sciences and Technology, ETH Zurich, Zurich, Switzerland

[b]Neuroscience Center Zurich, University and ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[c]Future Health Technologies, Singapore-ETH Centre, Campus for Research Excellence and Technological Enterprise (CREATE), 138602, Singapore.

*Address for correspondence: Sarah Nadine Meissner or Nicole Wenderoth, Neural Control of Movement Laboratory, Department of Health Sciences and Technology, ETH Zurich, Technoparkstrasse 1, 8005 Zurich, Switzerland, Email: sarah.meissner@hest.ethz.ch, nicole.wenderoth@hest.ethz.ch

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Abstract

The brain's state of arousal influences cognitive functioning and mental well-being. It is controlled by several neuromodulatory nuclei in the brainstem and, particularly, by the locus coeruleus (LC). The LC is the main source of noradrenaline (NA) in the central nervous system where it exerts powerful effects on neural processing and autonomic function. Here, we investigate whether human participants can gain volitional control of their brain's arousal state using a new neurofeedback approach which exploits the mechanism that the eye's pupil diameter provides an indirect readout of arousal if light conditions are controlled. We show that pupil-based neurofeedback training is essential for learning how to self-regulate pupil size. Once acquired, pupil self-regulation significantly modulates neuromodulatory brainstem centers involved in arousal control and particularly the LC-NA system when carefully measured with functional magnetic resonance imaging. Further, it modulates heart rate, a cardiovascular marker of autonomic function, and it has a significant effect on behavior and specific psychophysiological responses during an oddball task, an attention task that has been shown to be evoke stimulus-dependent LC-NA activity. Considering the modulatory effects of the LC-NA system and other arousal-regulating centers on cognitive functioning and various behaviors including stress-related responses, pupil-based neurofeedback has a tremendous potential to be translated to behavioral and clinical applications across various domains.

Introduction

The arousal state of the brain is controlled by several key neuromodulatory nuclei in the brainstem, most notably the noradrenergic (NA) locus coeruleus (LC), but also the dopaminergic substantia nigra/ventral tegmental area (SN/VTA), the serotonergic dorsal raphe nucleus (DRN) and the cholinergic (ACh) basal forebrain including the nucleus basalis of Meynert (NBM) [1–4]. The LC in particular, has received increasing attention during the last years [2,5,6] because it serves as the main source of NA to the brain and exerts powerful effects on neural processing through efferent projections to various cortical and subcortical brain regions [6,7]. Theories predominantly based on intracranial recordings in animals suggest that the LC-NA system modulates not only functional circuits related to wakefulness and sleep [5,8–10] but also cognitive processes relevant for task engagement and optimization of task performance via its effects on target areas of NA transmission [4,7,10–12]. More specifically, these theories postulate that LC neurons exhibit two different activity patterns, i.e., tonic and phasic discharge patterns. Tonic activity is thought to be closely correlated with the general arousal state of the brain (i.e., high LC tonic activity is associated with high arousal), whereas phasic, more burst-like discharge is proposed to facilitate behavioral responses to task-relevant processes [4]. Studies using simple attention tasks probing these task-relevant processes such as the classic two-stimulus oddball paradigm suggest that phasic activity is heavily dependent on levels of tonic activity. For instance, LC recordings in monkeys revealed that elevated levels of tonic LC activity were associated with decreased phasic activity in response to oddball stimuli and declines in task performance [13,14]. Crucially, besides its widespread efferents to almost the entire forebrain, the LC has (direct or indirect) projections to structures in the brainstem and spinal cord regions regulating autonomic functions including cardiovascular parameters (for an overview see [10]). Accordingly, changes in LC activity have been shown to cause delayed cardiovascular responses [15].

During the last years, a growing body of research indicated that, under constant lighting conditions, the eye's pupil size might be an indirect and non-invasive 'indicator' of the brain's arousal state. Arousal-related neuromodulatory systems have been directly or indirectly linked to these non-luminance-related changes in pupil size [16–18], however, strongest evidence has been obtained for the LC-NA system to be implicated in pupil control [18–22]. For instance, correlational evidence in humans has been provided by simultaneous acquisition of functional magnetic resonance imaging (fMRI) and pupillometry that has linked LC activity to modulation of pupil size, both at rest and during various emotional or cognitive tasks including the oddball paradigm [17,19,20,23]. More causally, selective chemogenetic or optogenetic activation of the LC in mice causes substantial pupil dilation [22,24,25] in parallel with modulated neural activity in specific brain networks related to threat detection and altered behavior indicative of increased anxiety [22]. Even though a direct comparison of cholinergic and noradrenergic systems and their role in pupil dynamics in rodents provide evidence for the involvement of both systems, only longer-lasting dilations of the pupil, such as during

locomotion, were accompanied by sustained cholinergic activity, while moment-to-moment pupil fluctuations during quiet rest closely tracked noradrenergic activity [18]. Furthermore, noradrenergic activity has been observed to precede cholinergic activity relative to peak pupil dilation [18] and can even depolarize cholinergic neurons [1], raising the possibility that noradrenergic activity drives cholinergic activation. Anatomically, the size of the pupil is controlled by the tone of (i) the dilator pupillae muscle receiving innervation via noradrenergic sympathetic neurons and (ii) the constrictor pupillae muscle receiving innervation via cholinergic parasympathetic neurons [10]. Thus, pupil diameter reflects the relative activity of these two opposing outputs of the autonomic nervous system (ANS; [6,10]). One assumption is that changes independent of luminance are mediated by the activity of the LC by a facilitation of sympathetic activity and modulation of the parasympathetic pathway via the Edinger-Westphal nucleus (EWN) in the midbrain ([6,10] for a review, see [26]). Similarly, there is first evidence that the superior colliculus (SC), a second hub in the brainstem of a network controlling pupil responses related to the integration of multisensory input and orienting responses, may influence parasympathetic (i.e., via the EWN) and sympathetic pathways via direct or indirect projections [21,26]. Whether the link between pupil size and other brainstem nuclei and neuromodulatory systems including the NBM are triggered directly or are rather routed through the LC-NA system is, however, still an ongoing debate [16,26].

Until today, regulating LC-mediated arousal is challenging and existing approaches in humans are mainly limited to systemic pharmacological agents that often cause pronounced side-effects. Here, we investigated an alternative approach which seeks to exploit the mechanistic link between LC-NA-mediated arousal regulation and pupil diameter via a novel pupil-based neurofeedback approach (pupil-NF). So far, only a few studies have trained volunteers to self-regulate pupil size with varying degrees of success [27–30]. Particularly volitional downregulation of pupil size is difficult to acquire [30]. The main idea of our approach is that participants apply different arousing or relaxing mental strategies while receiving quasi-online pupil-NF (Figure 1A). We hypothesized that (i) this type of pupil-NF would allow them to discover suitable mental strategies for volitional up- versus downregulation of pupil size and (ii) that such self-regulation is associated with up- versus downregulating activity in LC and (interconnected) areas involved in arousal control rather than brainstem areas involved in controlling pupil size in the context of the orienting response such as the superior colliculus (SC) [26,31–33]. We further hypothesized that self-regulating LC activity will (iii) modulate stimulus detection behavior during an oddball task (which has been shown to strongly depend on LC-NA activity) and (iv) cause systematic changes in cardiovascular parameters.

Numerous studies have shown that providing feedback on specific body functions including brain activity via neuro- or biofeedback enables humans to gain remarkable control over these functions [34–37]. The effectiveness of self-regulation critically depends on whether participants can acquire a suitable mental strategy. Neurofeedback has been shown to significantly facilitate this process [34,37].

However, the quality of the feedback signal and whether it provides an accurate representation of the to-be-modulated system, is a key determinant of utility. Using our new pupil-NF training regime, we show here that healthy volunteers can learn to volitionally up- and downregulate their pupil size (Experiment 1A,1B). Crucially, by combining pupil self-regulation with fMRI and measurements of cardiovascular parameters, we showed that up- versus downregulating pupil size systematically modulates (i) activity of LC and (interconnected) cortical and subcortical brain regions, including the SN/VTA and NBM (Experiment 2) and (ii) heart rate (Experiment 1B,2). Finally, we demonstrated that self-regulating pupil size modulates behavioral performance and psychophysiological markers of LC-NA activity during the classic two-stimulus auditory oddball paradigm (Experiment 3).

Results

Experiment 1: Pupil-NF training enables healthy volunteers to volitionally regulate pupil size

Healthy participants were randomly assigned to either a pupil-NF (n = 28) or control group (n = 28) and participated in three days of pupil-NF training (Experiment 1A). At the beginning of the training, all participants received instructions on potential mental strategies that were derived from previous research [27,30,38–40], such as imagining emotional (i.e., stressful, fearful or joyful) situations to upregulate pupil size (UP), or relaxing, safe situations together with focusing on their body and breathing to downregulate pupil size (DOWN). Then they participated in three training sessions on three separate days (Figure 1B), each consisting of 30 UP trials and 30 DOWN trials which required them to self-regulate pupil for 15s (Figure 1C, lower panel). Participants of the pupil-NF group received isoluminant visual feedback on pupil size in quasi real-time during the trial and average performance feedback after the trial (post-trial feeedback; Figure 1C upper panel). Participants of the control group saw matched visual information on the screen that, however, they did not receive veridical feedback on pupil size. At the end of training day 3, all participants underwent an additional *no feedback session* (20 UP and 20 DOWN trials) during which they used the same self-regulation strategies as during the actual training, but without receiving feedback on pupil size.

Participants of both groups (n = 27 in each group for final analyses) were able to volitionally upregulate their pupil size (Figure 2A), but the amount of upregulation was substantially larger in the pupil NF group than in the control group. The ability to downregulate pupil size became gradually more successful during training in the pupil-NF group while this ability was strongly reduced in the control group (Figure 2B). Importantly, participants of the pupil-NF group maintained their self-regulation abilities also during no feedback trials at the end of the training, indicating that they have acquired a transferable skill that, once learned, can be applied without the need for constant feedback (Figure 2A, B; grey lines).

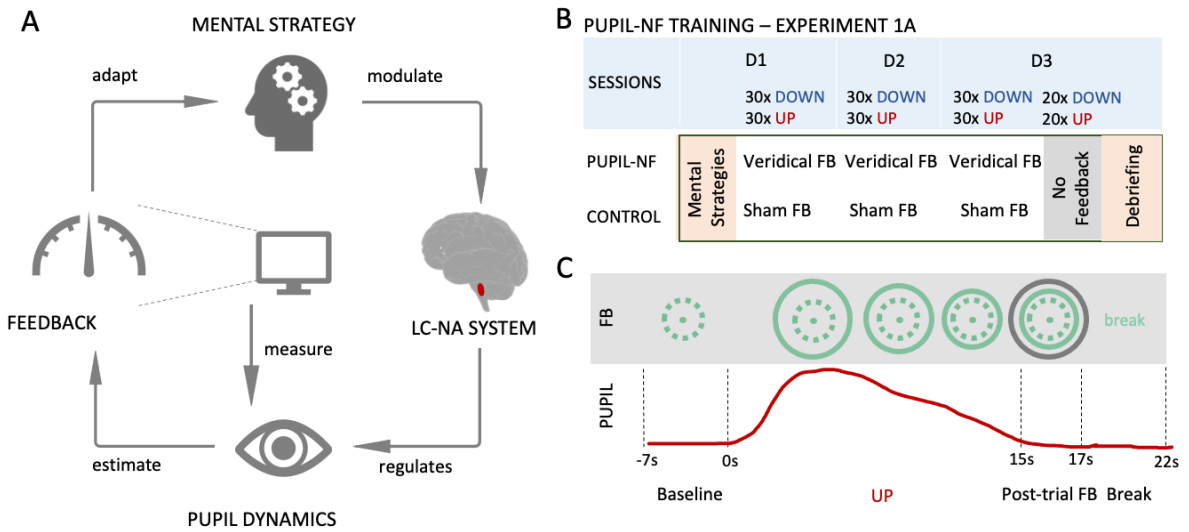

Figure 1. Pupil-based neurofeedback (pupil-NF) training. **A.** Participants apply mental strategies which are believed to modulate arousal via the LC-NA system. Pupil size is measured by an eye tracker and fed back to the participant via an isoluminant visual display **B.** In Experiment 1A, healthy volunteers were informed about potential mental strategies of arousal regulation and then participated in 3 days (D1, D2, D3) of up-regulation (UP) and downregulation (DOWN) training (30 trails each) while receiving either veridical pupil feedback (pupil-NF group) or visually matched sham feedback (control group). At the end of day 3, all participants performed 20 UP and DOWN trials without receiving any feedback and indicated used strategies during debriefing. **C.** Each trial consisted of (i) 7s baseline measurements, (ii) 15s modulation phase where the pupil-NF group sees a circle that dynamically changes its diameter as function of pupil size (veridical feedback), (iii) 2s of color-coded post-trial performance feedback (green = average circle size during veridical feedback; black = maximum (UP) or minimum (DOWN) circle size during veridical feedback), and (iv) 5s break. Note that the control group also sees a circle which, however, changes independently of their pupil size and resemble those of a participant of the pupil-NF group (sham feedback).

Pupil self-regulation was further quantified by calculating a pupil modulation index (UP – DOWN; i.e., the difference between the pupil diameter changes in the two conditions) which was significantly larger in the pupil-NF group than in the control group (Figure 2C; robust ANOVA main effect of *group:* $F(1,21.58) = 21.49$; $p = .001, \eta_p^2 = 0.50$; other main effects or interaction $p \geq .07$). A control analysis confirmed that these differences between the pupil-NF and control group were driven by volitional pupil modulation and not by differences in absolute pupil size at baseline (all $p \geq .49$; Supplementary Figure 1A).

Debriefing following training revealed that most participants of both groups imagined an emotional situation (sometimes in combination with imagining physical activity) in the UP condition and a relaxing situation (i.e., doing nothing, falling asleep, concentrate on breathing and how the body feels during breathing) in the DOWN condition.

Next, we conceptually replicated the above results in an independent pupil-NF cohort in Experiment 1B. 26 participants (n = 25 for final analyses) followed a similar 3-day training regime but participated in a no feedback session *before* (Pre) and *after* (Post) pupil-NF training. We calculated a modulation index time series (i.e. pupil changes UP – pupil changes DOWN across the 15s modulation phase) and found that pupil-NF training resulted in significantly stronger modulation of pupil diameter Post as compared to Pre pupil-NF training during most of the modulation phase (significant Pre versus Post differences were observed from 2.9s after modulation onset until the end of modulation; all $p < .049$; Bonferroni corrected, Figure 2D; for a condition effect see Supplementary Figure 2). This indicates that participants significantly improved their ability to volitionally modulate pupil size after pupil-NF training.

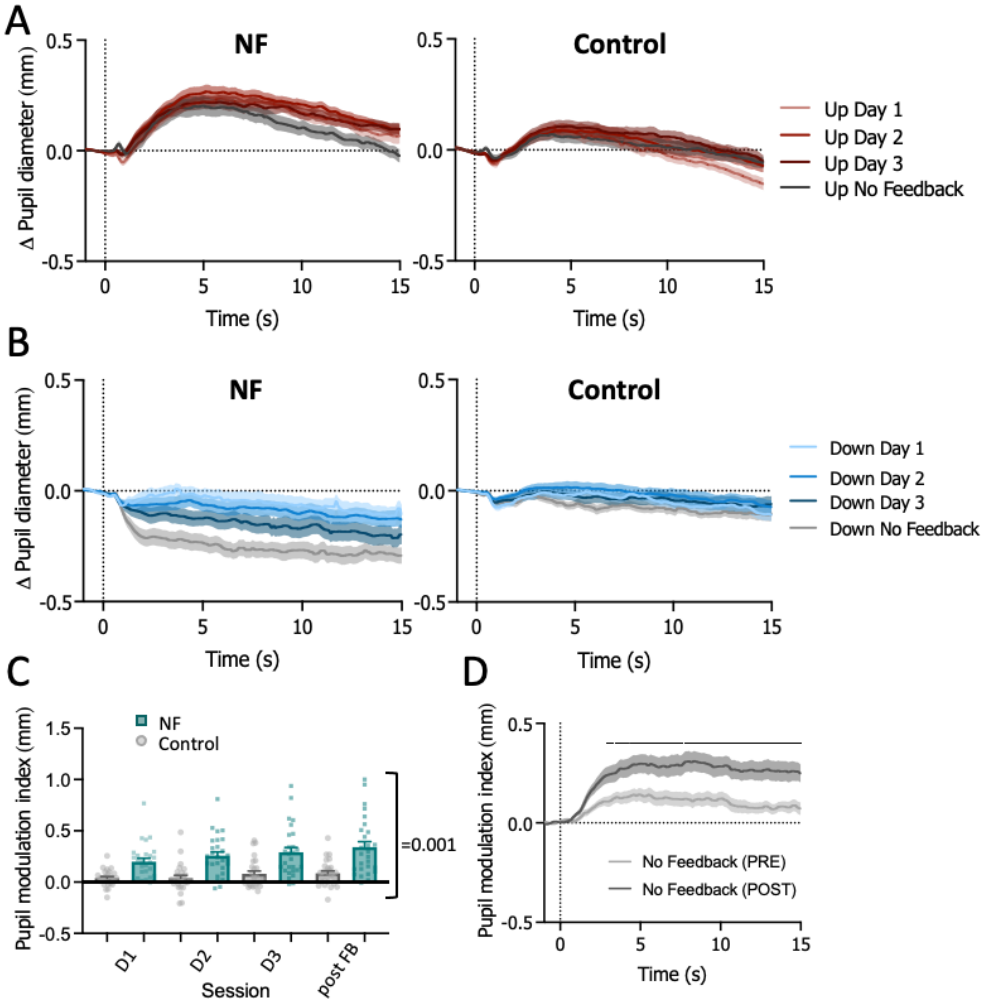

Figure 2. Average changes in pupil size during 15s upregulation (UP) **(A)** and downregulation (DOWN) **(B)** are shown for the pupil-NF (n=27) and the control group (n=27) for the training sessions on Day 1, Day 2, Day 3, and the no feedback post session of Experiment 1A. **C.** The pupil modulation index reflects the difference between the average pupil size during the two conditions (UP – DOWN) and is shown for each session (Day 1, Day 2, Day 3, no feedback session post training) and group (control versus pupil-NF group of Experiment 1A; dots and squares represent individual participants). Pupil modulation indices were generally higher in the pupil-

NF group as compared to the control group (main effect of *group*: $F(1,21.58) = 21.49$; $p = .001$, $\eta_p^2 = 0.50$). **D.** Time series of pupil modulation index measured during the no feedback session before (Pre, light grey) and after pupil-NF training (Post, dark grey) from Experiment 1B (independent cohort, n = 25). Solid black lines at the top indicate clusters of significantly higher modulation indices at Post compared to Pre (SPM1D repeated measures ANOVA; all $p < .05$; Bonferroni-corrected). Shaded areas and error bars indicate SEM.

In summary, our pupil-NF training results from Experiment 1A and B show that healthy volunteers can learn to volitionally modulate their own pupil size. This acquired self-regulation seems to be a transferable skill that – once acquired and optimized – is no longer dependent on feedback. Importantly, veridical neurofeedback significantly improves volitional control of pupil diameter as indicated by substantial differences in self-regulation capabilities revealed by comparing a pupil-NF group versus a matched control group and Pre versus Post performance of a separate pupil-NF cohort.

Experiment 2: Combining self-regulation of pupil size with simultaneous fMRI

Previous studies have repeatedly linked non-luminance-mediated changes in pupil size during rest or cognitive tasks to activity changes in arousal-regulating centers such as the LC [17,19–22]. Thus, in a next step, we tested the hypothesis that self-regulating pupil size is associated with activity changes in the LC and other interconnected brain regions involved in arousal-regulation. 25 trained participants of the pupil-NF groups from Experiment 1 performed pupil up- versus downregulation during two fMRI sessions, one measuring neural activity across the whole brain, one specifically measuring brainstem activity (the order of these sessions was counterbalanced). Participants performed 15s of volitional pupil size modulation and received post-trial feedback about their performance.

Pupillometry data obtained during the fMRI sessions confirmed that participants were able to successfully self-regulate pupil size (Figure 3A, 4A).

Self-regulation of pupil size is linked to changes in LC activity and other brainstem regions involved in arousal and autonomic regulation

After applying rigorous pre-processing steps to remove physiological noise from brainstem data (for details see Methods section Experiment 2), we investigated whether pre-defined areas of the brainstem and basal forebrain that have been linked to arousal or pupil size regulation were modulated by up- versus downregulation of pupil size (n = 22). According to our anatomical a-priori hypothesis, we extracted the mean signal intensity during UP > rest and DOWN > rest from the LC, the dopaminergic SN/VTA, the serotonergic DRN, the cholinergic NBM, and the SC (Figure 3B, F). As hypothesized, we observed that pupil upregulation was associated with significantly higher activation in the LC than pupil downregulation (Wilcoxon signed rank tests; $Z = 3.0$, $p = 0.015$, $r = 0.64$). Similar results were found for the SN/VTA ($Z = 2.90$, $p = 0.016$, $r = 0.62$) and a trend-level for the NBM ($Z = 2.26$, $p =$

0.072, r = 0.48; all *p*-values are sequential Bonferroni-corrected [41]; Figure 3B). Activation differences in all other ROIs failed to reach significance ($p \geq .32$) and an additional post-hoc Bayesian analysis revealed moderate evidence for the null hypothesis (i.e., UP = DOWN, Bayesian paired samples t-test for SC: BF10 = 0.34; error percentage = 0.03 %; DRN: BF10 = 0.31; error percentage = 0.02%).

We further investigated whether pupil size modulations recorded throughout the fMRI run correlated with continuous BOLD activity changes in the pre-defined brainstem ROIs and found a significant association in the SN/VTA (Z = 3.72, $p = .005$, r = 0.79), LC and NBM (trend-level after correction for multiple comparisons; LC: Z = 2.22, $p = 0.078$, r = 0.47; NBM: Z = 2.39; $p = 0.068$, r = 0.51) but not in the DRN or SC (all $p \geq 0.16$; Figure 3C).

Next, we complemented our a-priori-defined ROI analysis with a data-driven approach considering activity changes for UP > DOWN across the whole brainstem. Given the limited signal-to-noise ratio for brainstem responses and our strict pre-processing pipeline, we report all voxels reaching significance at $p < 0.05$ without applying corrections for multiple comparisons (for a similar approach, Esee [23]). In line with our ROI analyses, we identified significant bilateral activation in the LC (Figure 3D, upper panel), SN, VTA, and NBM (Figure 3E, upper panel). Additionally, we identified small activity clusters overlaying the SC and the DRN (just at the very caudal part; Figure 3E; upper panel). Importantly, pupil upregulation activated several brainstem nuclei involved in arousal and autonomic regulation [42–48] such as the pedunculopontine nucleus, one of the main components of the Ascending Reticular Activating System (ARAS), the parabrachial nucleus, and the periaqueductal gray.

We also correlated continuous changes in pupil diameter with continuous changes in BOLD activity for every voxel in the brainstem. Confirming our previous results, we found a significant correlation between pupil size and BOLD activity at the LC (Figure 3D, lower panel), SN, VTA, the NBM, and a rather small overlap at the caudal part of the DRN (Figure 3E, lower panel). Additionally, pupil size was found to correlate with activity in brainstem regions involved in arousal and autonomic regulation [42–48] similar to the UP > DOWN contrast.

Investigating specific activity patterns for downregulation (reverse contrast of DOWN > UP), we only found activation clusters in white matter tracts of the brainstem (i.e., the pontine crossing tract).

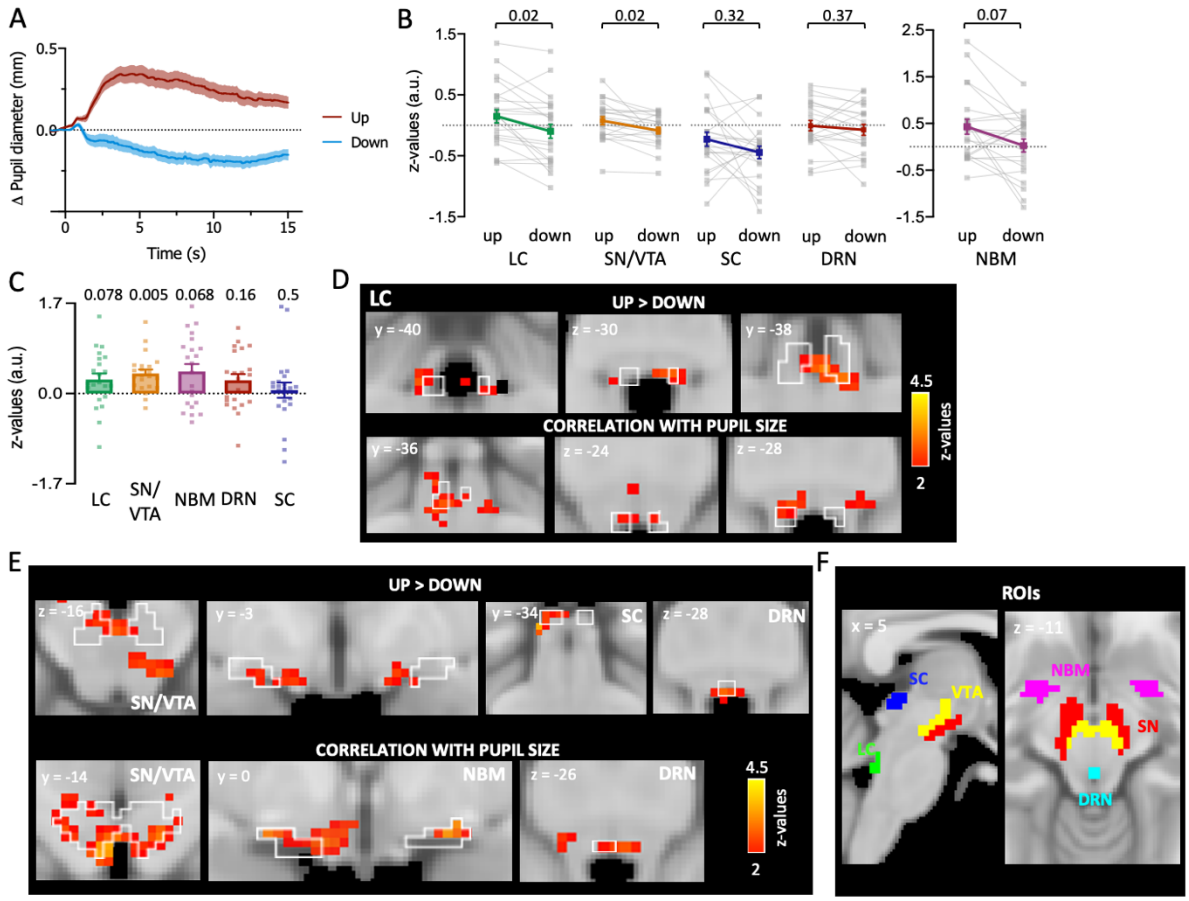

Figure 3. Brainstem fMRI session. **A.** Changes in pupil size averaged across all participants for UP (red) and DOWN (blue) trials. **B.** Activity during UP versus DOWN phases of pupil self-regulation in the different region of interests (ROIs). Statistical comparisons revealed significant effects (UP > DOWN) in the LC, the SN/VTA, and the NBM (trend-level), but not in the SC and DRN. **C.** Correlation between continuous pupil size changes and BOLD response changes shown as Z-Values for the different ROIs. Statistical comparison (against 0) revealed significant effects for the SN/VTA as well as trend-level significance for the LC and the NBM. All ROI analyses in **B** and **C** were corrected for multiple comparisons using sequential Bonferroni correction. Squares represent individual data **D. Upper panel:** Data-driven analysis across the brainstem revealed a significant bilateral activation cluster at the LC during pupil upregulation (UP > DOWN; $p < .05$, uncorrected; upper panel). **Lower panel:** Correlation analysis revealed that BOLD activity at the LC covaries with continuous changes in pupil diameter ($p < .05$, uncorrected). **E. Upper panel:** Other brainstem areas than LC were identified by the data-driven analysis when comparing UP > DOWN regulation of pupil size. Significant clusters ($p < .05$, uncorrected) were found in SN/VTA, NBM, and small parts of the SC and DRN. **Lower panel:** The same areas – except for the SC – exhibited a significant correlation between changes in pupil diameter and BOLD activity (lower panel; $p < .05$, uncorrected). The white outlines in **D** and **E** indicate different brainstem [42–48] and basal forebrain regions [49]. **F.** A-priori defined ROIs in the brainstem and basal forebrain in MNI space. Shaded areas and error bars indicate SEM. Locus coeruleus (LC); substantia nigra (SN) and ventral tegmental area (VTA) combined to one ROI (SN/VTA); dorsal raphe nucleus (DRN), nucleus basalis of Meynert (NBM); and superior colliculus (SC).

In summary, our brainstem fMRI analyses revealed that pupil self-regulation is indeed linked to changes in activity in the LC driven by activation during upregulation of pupil size and deactivation during downregulation of pupil size. Not surprisingly, LC is not the only area that was modulated by pupil self-regulation, but we also identified the dopaminergic SN/VTA, the cholinergic NBM and other brainstem regions that are known to be involved in arousal and autonomic regulation and are closely interconnected with the LC.

Self-regulation of pupil size is linked to changes in cortical and subcortical regions closely connected with the LC

To be able to investigate the effects of self-regulated pupil size on a wide range of cortical and subcortical structures, we analyzed whole-brain fMRI data (n = 24). Contrasting UP > DOWN phases, we found that upregulation was associated with significantly more activation in various brain regions known to be closely connected to the LC, most notably in the dorsal anterior cingulate cortex (ACC), the paracingulate gyrus (PCG), the dorsolateral prefrontal (DLPFC) and orbitofrontal cortex (OFC), the precuneus and the thalamus (Figure 4C, D; Supplementary Table 1A). Additionally, we observed significant activation in primary somatosensory regions (SM1), the basal ganglia including the globus pallidus and caudate nucleus, and the cerebellum (i.e., in VI and Crus I; Figure 4C, D).

Next, we investigated in which brain areas BOLD activity covaried with pupil size throughout the task and observed significant effects in a cortico-subcortical network including the dorsal ACC, thalamus, globus pallidus, and in the cerebellum (Figure 4B; Supplementary Table 2), partially overlapping with the regions significantly activated during upregulation of pupil size (UP > DOWN).

The visual area V5 was the only region where BOLD activity was significantly higher during DOWN compared to UP, (Figure 4C, Supplementary Table 1B), an effect that was driven by a deactivation during the upregulation period (Figure 4E).

Taken together, our whole-brain fMRI data indicates that pupil self-regulation is linked to an interplay of activation and deactivation in a set of circumscribed brain regions including prefrontal, parietal, thalamic and cerebellar areas strongly interconnected with the LC in the brainstem.

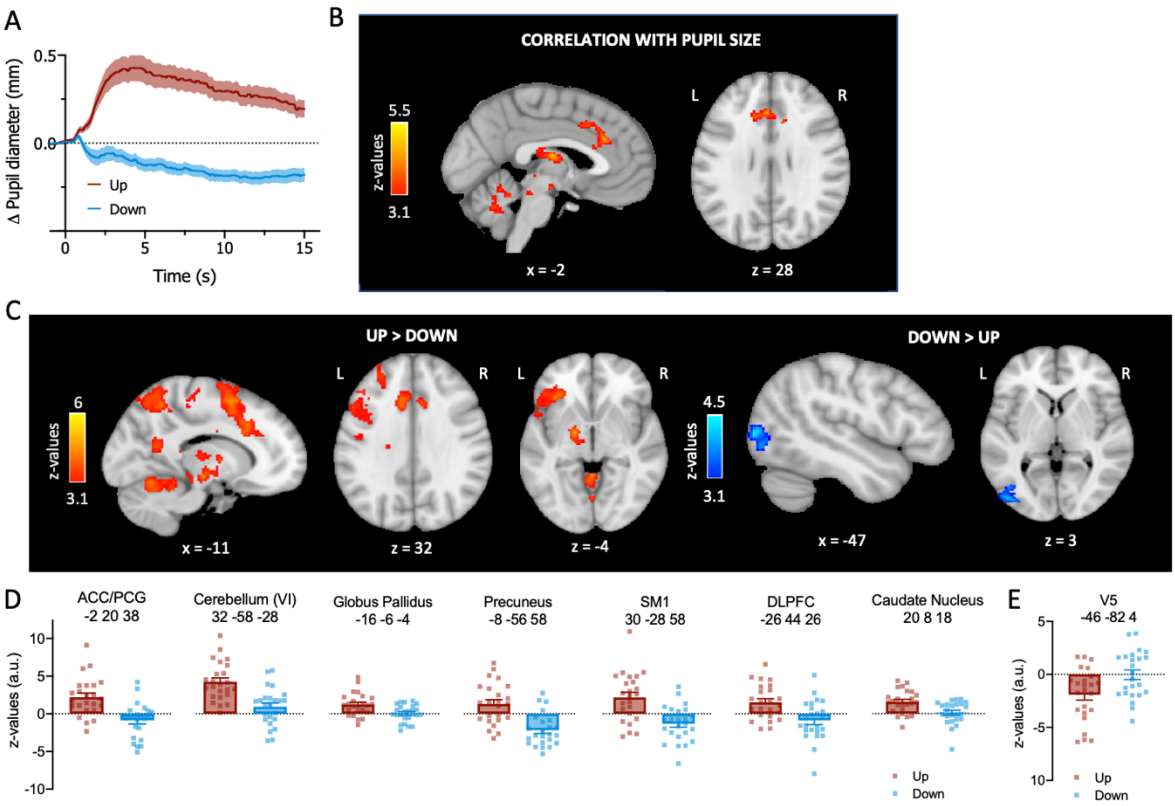

Figure 4. Whole-brain fMRI results. **A.** Changes in pupil size averaged across all participants for UP (red) and DOWN (blue) trials showing successful self-regulation of pupil size during whole-brain fMRI recordings. **B.** Whole-brain maps showing brain regions where BOLD activity correlates with pupil size changes throughout the run **C.** Whole-brain maps depicting brain regions that showed a significant activation during UP as compared to DOWN (left panel, shown in red) and during DOWN as compared to UP (right panel, shown in blue). All activation maps are thresholded at Z > 3.1 and FWE-corrected using a cluster significance level of $p < .05$. **D.** and **E.** Estimated BOLD response represented by z-values for UP vs rest and DOWN vs rest extracted from the peak voxel of each significant cluster shown in C. Squares indicate individual participants. Shaded areas and error bars indicate SEM.

Self-regulation of pupil size modulates cardiovascular parameters

We tested whether self-regulating pupil size influenced cardiovascular parameters by analyzing electrocardiogram (ECG) signals recorded in 15 participants during pupil-NF training (Experiment 1B) and pulse oximetry data recorded during the subsequent fMRI sessions (Experiment 2). Generally, we observed higher heart rates during UP as compared to DOWN trials (Figure 5A, C). This difference became increasingly pronounced throughout pupil-NF training (repeated measures ANOVA on training data: *condition* x *session* interaction; $F(2.35, 32.89) = 3.98$, $p = .023$, $\eta_p^2 = 0.22$; Greenhouse-Geisser-corrected) and remained stable post-training during fMRI (repeated measures ANOVA on fMRI data: main effect *condition*; $F(1,22) = 72.25$, $p < .001$, $\eta_p^2 = 0.77$; Figure 5A,C). We further showed that changes in pupil diameter and changes in heart rate were largely unrelated prior to pupil-

NF training ($p > 0.5$, Figure 5E, left panel) but exhibited a significant positive correlation after training during fMRI where a higher pupil modulation index (UP-DOWN), thus better pupil self-regulation, was associated with larger differences in heart rate (UP-DOWN; rho = 0.53; $p = .02$; Bonferroni-Holm corrected; Figure 5E, right panel).

We also calculated the root mean square of successive differences (RMSSD), a heart rate variability (HRV) measure suggested to represent a marker of parasympathetic activity. We chose RMSSD because it is relatively free from breathing influences [50] and can be computed for intervals as short as 10s [51,52]. There was no clear effect of pupil self-regulation training on HRV (robust ANOVA on training data: all $p > 0.34$; left panel; Figure 5B, D) and changes in pupil diameter were largely unrelated to HRV changes prior to and following training (i.e., during fMRI; all $p > 0.2$, Figure 5F). However, during the fMRI session, HRV was generally higher during down- than upregulation of pupil size which nearly reached significance (Wilcoxon Signed Rank Test; brainstem session: Z = 1.71, $p = 0.086$, r = 0.35; whole-brain session: Z = 1.91; $p = 0.056$, r = 0.39; right panel, Figure 5B, D).

Taken together, pupil self-regulation following pupil-NF training modulated cardiovascular parameters. Interestingly, such modulation was more pronounced and consistent for heart rate as compared to HRV.

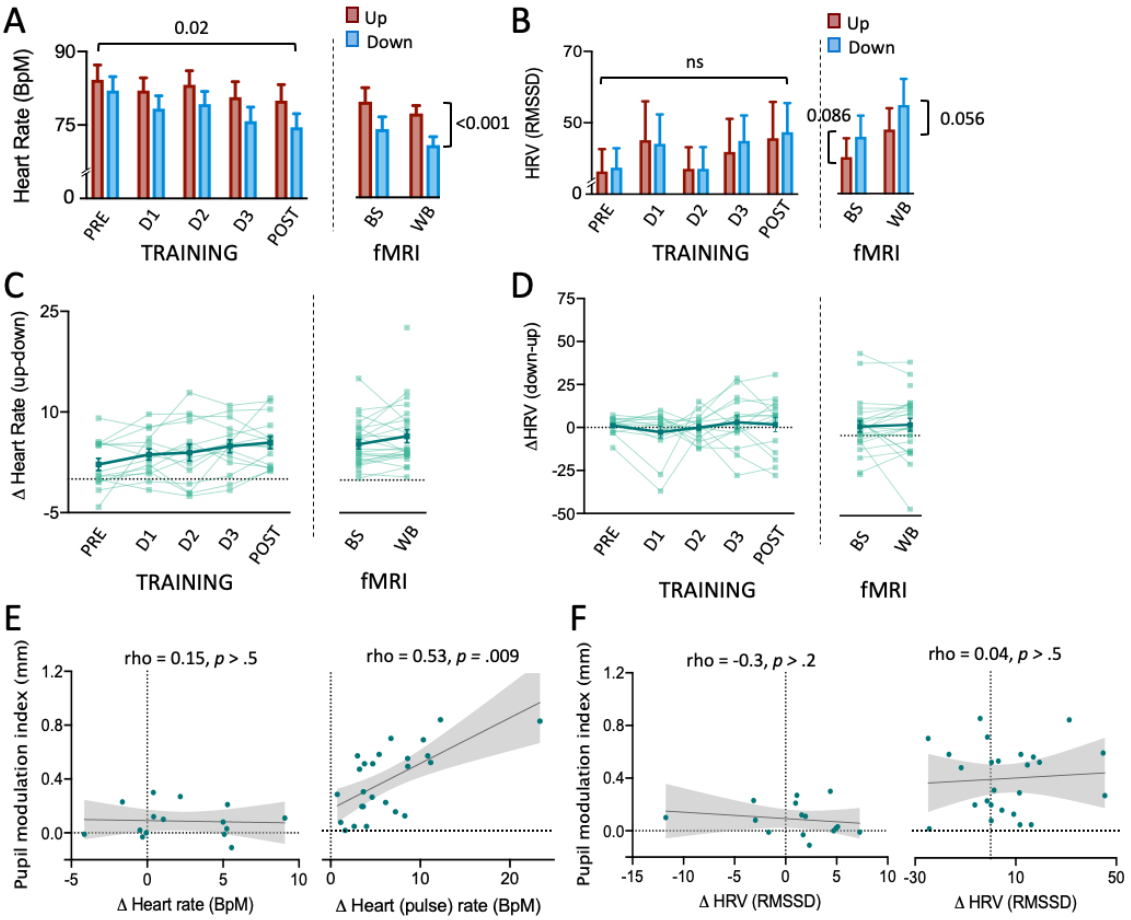

Figure 5. Effects of pupil self-regulation on cardiovascular parameters. Heart rate **(A)** and heart rate variability (HRV) **(B)** averaged for UP and DOWN trials across all participants for pupil-NF training (n = 15, left panel) and fMRI sessions (n = 24, right panel). HRV was estimated as root mean square of successive differences (RMSSD). Self-regulation of pupil size systematically modulated heart rate with an increasingly larger difference between UP and DOWN trials over training sessions (*condition* x *session* interaction; $p = .02$) which remained stable after training during fMRI (main effect *condition;* $p < .001$). Self-regulation did not significantly modulate HRV during training ($p > 0.34$). After training during fMRI, we found a trend-level effect for higher HRV during DOWN than UP (brainstem: $p = 0.086$; whole-brain: $p = 0.056$). **C.** and **D.** show individual differences in heart rate (UP-DOWN differences) and RMSSD (DOWN-UP differences) for training (left panels) and fMRI sessions (right panel). The thick solid line represents the group average, thin lines represent individual data. **E.** Spearman rho correlation coefficients between pupil modulation indices (i.e., the difference between pupil diameter changes in the two conditions; UP-DOWN) and differences in heart rate (UP-DOWN) revealing a significant link following (right panel; during fMRI) but not prior to pupil-NF training (left panel) **F.** Non-significant Spearman rho correlation coefficients between the pupil modulation index (UP-DOWN) and RMSSD differences (DOWN-UP) prior to (left panel) and after pupil-NF training during fMRI (right panel). Error bars indicate SEM. The grey shading (**E., F.)** around the solid line represents the 95% confidence interval around the line of best fit. BS = brainstem fMRI; WB = whole-brain fMRI.

Experiment 3: Combining self-regulation of pupil size with an auditory oddball task

To determine whether self-regulation of pupil size further impacts behavioral measures and psychophysiological markers of LC-NA activity, we combined our pupil-NF approach with a classic two-stimulus auditory oddball task (Figure 6A). The oddball paradigm is a task during which the detection of salient target stimuli has been linked to transient, phasic LC activity [53] that is assumed to significantly modulate neural processing in cortical target areas [4,11,12]. 22 participants that had undergone pupil-NF training (as part of Experiment 1A, B) performed an oddball task while (i) up-regulating pupil size (ii) down-regulating pupil size or (iii) executing a cognitive control task which required them to silently count backwards in steps of seven. This latter condition was included to control for effects of cognitive effort that might arise from executing pupil-self regulation simultaneously with the oddball task.

Participants (n = 20 for final analyses) were able to successfully self-regulate pupil size (robust repeated measures ANOVA; main effect *condition*: $F(1.52,16.67) = 9.33$, $p = 0.003$, $\eta_p^2 = 046$; Figure 6B, C) as indicated by smaller (baseline-corrected) pupil sizes during DOWN as compared to UP and control trials, even when simultaneously performing the oddball task. However, comparing pupil size changes during UP to control trials only led to tendentially significant differences ($p = .06$) indicating that successful upregulation seems to be more difficult when being engaged in another cognitive task. Further, calculating absolute pupil size time series during the baseline and self-regulation phases revealed generally larger absolute pupil sizes during control as compared to UP and DOWN trials

suggesting that performing a cognitive task such as counting also influences pupil size with a potentially higher but relatively stable cognitive load throughout the control condition (Supplementary Figure 3).

Self-regulation of pupil size modulates psychophysiological markers of LC activity during the oddball task

Previous research has shown that elevated levels of "consistent", tonic LC activity may constrain the intensity of more transient, phasic LC activity [13,14]. For instance, in LC recordings in monkeys, higher tonic activity was linked to decreased phasic activity in response to salient stimuli during an attention task [13]. Here, we tested the prediction that phasic activity varies depending on levels of tonic activity. Particularly, we investigated whether sustained volitional pupil size modulation would impact on pupil dilation responses to target sounds during the simultaneously performed oddball task which have been linked to phasic LC activity. In line with previous literature, self-regulation of pupil size prior to the target tone led to differences in pupil dilation responses to target sounds (significant cluster main effect condition: (z^* = 7.29): 714-3000ms, p < .001, Figure 6D, left panel) with significantly larger initial pupil dilation and prolonged elevated dilation levels during DOWN as compared to UP trials (significant clusters post-hoc tests (z^* = 4.07): 718-2669ms, p < 001; 2851-3000ms, p = .005, Bonferroni-corrected).

The cognitive control condition caused a similar initial pupil dilation as the UP condition but the time course of subsequent pupil constriction between 1381-3000ms differed significantly between Control and UP trials (several significant clusters between 1381ms-3000ms after playing the target tone, all p ≤ .02; Bonferroni-corrected, Figure 6D left panel). Differences between control and DOWN trials did not reach significance, even though the initial pupil dilation response was descriptively larger in the DOWN versus control condition (Figure 6D left panel). Finally, we tested whether there is a systematic relationship between baseline pupil size and phasic pupil response. We found that smaller baseline pupil sizes before target onset were significantly correlated with larger pupil dilation responses towards the target sound (repeated measures correlation r_{rm} = -0.28, p < .001; Figure 6F left panel).

Standard tones evoked only a minor pupil response (Figure 6D, right panel). Responses differed significantly between conditions mainly driven by prolonged elevation of pupil size in UP than DOWN trials (significant cluster (z^* = 3.93): 1081-2251ms, p < 001; Bonferroni-corrected) and a faster decrease in pupil size in DOWN than control trials (significant clusters (z^* = 4.23), between 916-1233ms, all p ≤ .02; Bonferroni-corrected). However, since these differences were observed mainly *after* peak dilation and in the opposite direction as observed during dilation to target sounds, they likely reflect effects of self-regulation rather than modulation of pupil dilation responses to standard sounds.

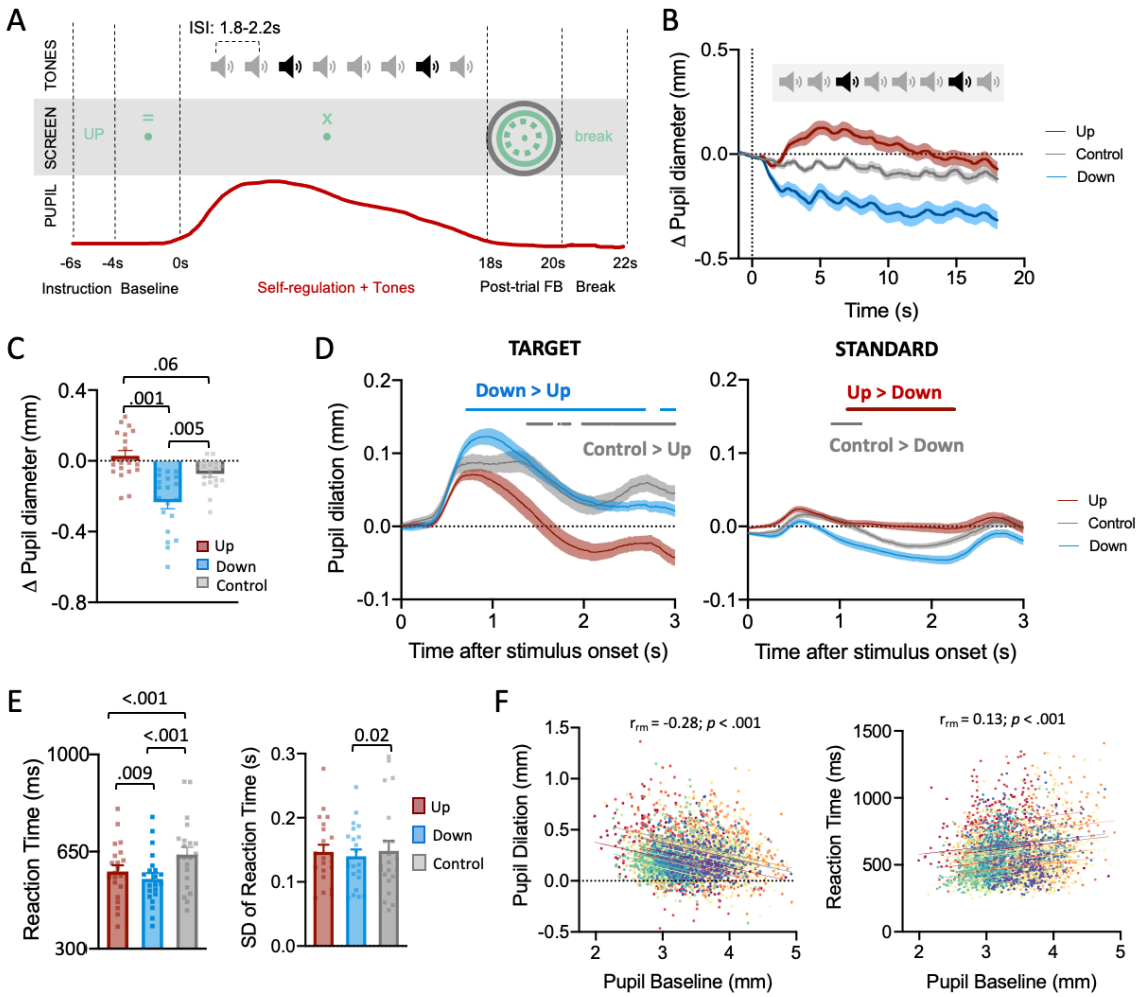

Figure 6. Self-regulation of pupil size combined with an auditory oddball task. **A.** Schematic depiction of an example trial (UP) of Experiment 3 combining pupil-NF and the oddball task in which participants reacted to target tones (black sound icon) by button press and ignored standard tones (grey sound icon) while simultaneously up- or downregulating pupil size or counting backwards (i.e., control). **B.** Pupil size changes averaged across participants for UP (red), DOWN (blue) and control trials (grey, counting backwards in steps of 7) showing the last second of the baseline phase as well as the complete modulation phase of 18s for 20 participants. **C.** Pupil size changes during the modulation phase from baseline (last 1s of baseline phase) averaged across the respective conditions and participants showing significantly lower values in DOWN as compared to control and UP trials (robust repeated measures ANOVA with the factor *condition:* $F(1.52,16.67) = 9.33$, $p = 0.003$, $\eta_p^2 = 046$; DOWN vs. UP: $\hat{\psi} = -0.23$; $p = .001$; DOWN vs. control: $\hat{\psi} = -0.14$; $p = .005$; for UP vs. control we only found a trend-level effect: $\hat{\psi} = 0.10$; $p = .06$). **D.** Baseline-corrected pupil dilation responses evoked by target (left) and standard tones (right) for UP, DOWN and control trials. Solid lines above the dilation time series indicate time windows of significantly smaller responses to targets in UP as compared to DOWN (blue) and control trials (grey; left panel) and significantly larger responses to standards in UP (red) and control (grey) than in DOWN trials (right panel; all tests are Bonferroni-corrected post-hoc tests of SPM1D repeated measures ANOVA with the factor *condition;* all $p < .05$) **E.** Behavioral performance of 21 participants during the oddball task depicting faster responses during DOWN as compared to UP ($t(20) = -2.87$, p = .009) and control trials (grey; $t(20) = -7.19$, $p < .001$; UP versus control: $t(20) = -6.04$, $p < .001$; left panel). Responses

were also less variable (right panel) in DOWN as compared to control trials ($t(20) = -3.01$, $p = .02$; sequential Bonferroni-corrected post-hoc tests of repeated measures ANOVA on reaction time and SD of reaction times with the factor *condition*). **F.** Results of repeated measures correlations between baseline pupil size (500ms prior to target onset) with pupil dilation responses (left panel) and reaction times (right panel) towards targets. Please note that sound icons in **A.** and **B.** indicate standards (grey) and targets (black) played during pupil self-regulation or control trials. Squares **C.** and **E.** represent individual data. Error bars and shaded areas indicate SEM.

Self-regulation of pupil size modulates performance during the oddball task

LC activity during the oddball task has been linked to the detection of task-relevant target stimuli [13,19,53]. Thus, we here tested the hypothesis that self-regulation of pupil size modulating LC activity will also influence behavioral performance during the oddball task. In general, task performance was high during the oddball task (correct responses for control trials: 94,3%, UP: 95.8%, DOWN: 97.9%). Analyzing reaction times to target tones, we found that participants responded slowest to target tones during the cognitive control condition and fastest during DOWN (Figure 6E left panel, repeated measures ANOVA: condition effect $F(2,40) = 35.97$, $p < .001$, $\eta_p^2 = 0.64$; DOWN versus UP: $t(20) = -2.87$, $p = .009$, d = 0.63; UP versus control: $t(20) = -6.04$, $p < .001$, d = 1.32; DOWN versus control: $t(20) = -7.19$, $p < .001$, d = 1.57; sequential Bonferroni-corrected for multiple comparisons). Evaluating the relationship between single-trial baseline pupil size prior to target tones and the reaction times to targets revealed a significant positive correlation, i.e., smaller pupil size at target onset is associated with faster responses to the target (repeated measures correlations $r_{rm} = 0.13$, $p < .001$, Figure 6F, right panel).

Finally, comparing task performance variability by means of the standard deviation (SD) of reaction times revealed a significant difference between conditions (repeated measures ANOVA; main effect *condition:* $F(2,40) = 4.84$, $p = .01$, $\eta_p^2 = 0.19$; Figure 6E right panel) which was mainly driven by less variable responses in the DOWN as compared to control trials ($t(20) = -3.01$, $p = .02$, d = 0.66; sequential Bonferroni-corrected).

Taken together, our results reveal that self-regulation of pupil size systematically modulates stimulus-evoked pupil dilation responses and behavioral performance during the oddball task. Smaller self-modulated pupil size led to increased pupil dilation and faster and less variable responses to task-relevant sounds.

Discussion

In a series of experiments, we tested whether participants could gain volitional control over their brain's state of arousal via a newly developed pupil-NF approach that we hypothesized to tap into the mechanistic link between LC-NA activity and pupil size. We found that healthy young adults can learn to self-regulate pupil size during a 3-day training with veridical pupil-NF but not with sham feedback

(Figure 2). Investigating the neural, physiological, and behavioral consequences of volitionally up- versus downregulated pupil size revealed three main findings: First, LC activity was significantly modulated during pupil self-regulation, together with connected arousal-regulating centers including the dopaminergic SN/VTA, and the cholinergic NBM (Figure 3). Second, we observed systematic changes in cardiovascular parameters (i.e., heart rate in particular, Figure 5) in line with previous findings showing that LC exerts a strong influence on the cardiovascular system [10,15,54]. Third, pupil-self-regulation significantly influenced task performance and a well-established psychophysiological readout evoked by salient, task-relevant stimuli during an oddball task which has been shown to reflect phasic activity of the LC-NA system (Figure 6).

Before discussing the results of the present study, it is important to stress that imaging the human brainstem – and the LC in particular – is extremely challenging due to the small sizes of the nuclei, high susceptibility to physiological noise, and lower signal-to-noise ratio than cortical signals. However, we are confident that our findings represent reliable results because we applied stringent noise control by means of ICA and PNM methods. Given the strong correlation between heart rate and pupil self-regulation, considering heart rate as a nuisance regressor during PNM may have additionally weakened our results and we therefore provide a rather conservative perspective. To not be overly conservative, we reported exploratory, data-driven brainstem results at uncorrected levels [23].

Previous research has shown that it is possible to gain volitional control over body and brain functions if bio- or neurofeedback is combined with suitable mental strategies and integrated into an appropriate task setting [34,36,37,55]. We showed that providing feedback on pupil diameter significantly enhanced the ability to volitionally up- versus downregulate pupil size when compared (i) within participants from pre- to post-training and (ii) between a veridical neurofeedback group and a sham neurofeedback group. Interestingly, upregulation of pupil size was already strong during the first neurofeedback session on day 1, while downregulation evolved only over the course of training. This is in line with previous reports [28,30], where only upregulating but not downregulating pupil diameter was successful when participants were in a resting state and trained only for one session [30]. Our results further suggest that once acquired and optimized, self-regulation of pupil size seemed to be a skill that can be controlled in a feedforward manner and is no longer dependent on receiving constant feedback. Even though we tested this ability only over short periods of time in a no-feedback phase immediately after training, previous studies showed lasting self-regulation of central nervous system activity providing evidence that this skill is retained over time [34].

We had hypothesized that volitional modulation of pupil size is linked to activity changes in brain nuclei regulating the brain's arousal levels and in the LC in particular. Intriguingly, our ROI analyses on brainstem fMRI data during pupil self-regulation confirmed this hypothesis and revealed that up- versus downregulation of pupil size was indeed associated with systematic BOLD activity changes in

the LC and that pupil diameter dynamics throughout a run correlated with BOLD activity changes in the LC (trend-level effect after correcting for multiple comparisons). However, self-regulating pupil diameter did not exclusively modulate LC activity but led to analogous BOLD activity changes in the dopaminergic SN/VTA and – less consistently – in the cholinergic NBM (trend-level effects). These results are in line with a recent study in humans that specifically linked noradrenergic, cholinergic, and dopaminergic activity to pupil responses during a cognitive task [17] and the close interactions between the LC, the cholinergic basal forebrain including the NBM, and the dopaminergic VTA that have been demonstrated in animal models [56–60]. More specifically, using retrograde tracing, studies in non-human primates and rodents revealed that noradrenergic fibers originating from the LC have a neuromodulatory effect on the VTA and that each LC innervates VTAs in both hemispheres to a similar extent [59]. Similarly, the SN receives NA-projections from the LC [60] suggesting that activation of the LC may not only have modulatory influences on the VTA but also on the SN. Unfortunately, based on our design and methodology, we cannot disentangle whether SN/VTA modulation during pupil self-regulation is directly achieved via top-down control mechanisms or rather indirectly via the LC.

Here, links between self-regulating pupil size and the cholinergic NBM reached only trend-level significance and were less consistent than with dopaminergic and noradrenergic regions. A direct comparison of cholinergic and noradrenergic systems and their role in pupil dynamics in mice provided correlative evidence for the involvement of both systems. However, only longer-lasting dilations of the pupil, such as during locomotion, were accompanied by sustained cholinergic activity, while moment-to-moment pupil fluctuations during quiet rest closely tracked noradrenergic activity [18]. Furthermore, noradrenergic activity preceded cholinergic activity relative to peak pupil dilation [18], which – together with the finding that noradrenergic neurons can depolarize cholinergic neurons [1] – has given rise to the assumption that noradrenergic activity may drive cholinergic activation. Unfortunately, the limited temporal resolution and indirect nature of fMRI makes it impossible to unravel effects on such a fine-grained timescale in the present study in humans and to directly compare it to more causally induced and directly obtained measures in animal models. Interestingly, self-regulating pupil-diameter, did not systematically modulate BOLD responses in the serotonergic DRN and SC, two brainstem regions that have been implicated in non-luminance-dependent pupil size changes [16,26]. The absence of an effect in SC is in line with the theory that SC modulates pupil mainly in the context of specific orienting responses towards salient events while LC modulates pupil in the context of cognition and arousal [31].

The fact that our general linear model analyses on brainstem fMRI data revealed additional activity clusters at other critical nodes for producing a waking state and regulating autonomic activity including the pedunculopontine, the lateral parabrachial nucleus, and the periaqueductal gray, further emphasizes that self-regulating pupil dynamics modulates a distributed brainstem network which has been related

to arousal regulation. It is, however, unclear whether these effects are driven directly via cortical and/or subcortical top-down control mechanisms or rather through the LC as major relay station. To ultimately disentangle whether the observed, seemingly synchronized up- and downregulation of different neuromodulatory systems and brainstem nuclei during pupil self-regulation is orchestrated indirectly via the LC, future research combining pupil-NF with pharmacological agents targeting different systems is inevitable.

Our study has also identified candidate areas that might exert top-down control of the arousal system in the brainstem. Up- as compared to downregulation of pupil size was associated with BOLD activity changes in brain regions heavily interconnected with the brainstem and with the LC in particular, including the anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), dorsolateral prefrontal cortex (DLPFC), the precuneus, thalamus, globus pallidus, and the cerebellum. Our analyses do not allow us to determine whether activity in these areas is causal for arousal modulation or rather its consequence. However, it is tempting to speculate that particularly frontal areas such as the ACC and OFC that have dense projections to the LC in non-human primates [4,61] form the brain's intrinsic control system of arousal and exert top-down control of LC activity.

Together, our fMRI results extend previous findings by showing that pupil size not only can be used as an indirect readout for arousal [17,19,20] but could even provide an active information channel for self-regulating activity in areas involved in arousal regulation. Our results implicate LC as an important but not the only brainstem area that is significantly modulated by pupil self-regulation.

Pupil dilation responses evoked by an oddball task have been suggested to represent a psychophysiological marker of phasic LC activity [4,62]. Additionally, well-known theories derived from work in animal models postulate that phasic LC activity in response to task-relevant events depends on tonic LC activity: when tonic LC activity is upregulated, phasic LC responses are weak; whereas when tonic LC activity is relatively lower, phasic LC responses are strong [4,63]. A similar relationship has been observed in human pupil measurements reporting an inverse relationship between naturally fluctuating baseline pupil size and transient pupil dilation responses [62,64,65]. In line with these previous results, we found that downregulating pupil diameter in a sustained way at baseline, led to larger pupil dilation in response to the target stimulus and to faster and more consistent reaction times. By contrast, upregulating pupil diameter at baseline led to smaller pupil dilation in response to the oddball stimulus and longer reaction times. These results are consistent with previous work, strongly suggesting that self-regulating pupil diameter modulates tonic LC activity. One challenge concerning our interpretation is that it is difficult to exclude other explanations for this relationship such as intrinsic properties of the pupil related to the structure of the eye and pupil musculature. Even though we did not run control experiments in the present study, two previous studies modulated baseline pupil size via different illuminance conditions and could show similar task-evoked

pupil dilation responses independent of illuminance modulation [62,66] ruling out anatomical factors and providing evidence for a link between different pupil dilation responses and brain (or LC) state reflected in baseline pupil size. Our behavioral findings further confirm previous studies reporting enhanced task performance with relatively low baseline pupil diameter, either when changes in baseline diameter are experimentally induced [67] or occur spontaneously [62] (however, see [64]) whereas high baseline pupil size assumed to reflect high tonic activity might be detrimental to task engagement and performance [13,53]. Taken together, our data supports the idea that the state of tonic LC activity reflected by baseline pupil size prior to target onset predicts the pupil dilation response, a peripheral marker of phasic LC responses, and attentional performance. Such an influence may be achieved via a modulation of ongoing processes in cortical target areas of NA projections facilitating adequate behavioral responses.

In our oddball task, we also implemented a control condition where participants had to count backwards in steps of seven while responding to target tones. To our surprise, absolute pupil size was substantially higher in this condition than during downregulation trials already at baseline suggesting that our counting control task was cognitively effortful leading to increased pupil diameter throughout the task. Despite these differences in pupil size, we did not find a significant difference in dilation responses to target sounds between these two conditions, at least not when comparing pupil dilation time series using Bonferroni correction. Furthermore, participants showed slowest and most variable responses to task-relevant sounds in this counting control trials. We can only speculate that these effects may have been because of high cognitive load leading to dual task costs when simultaneously counting and responding to target sounds. These dual task costs may have been reduced for self-regulation trials since participants had been practicing this skill over several days resulting in more automatized processes.

We also investigated whether self-regulating pupil diameter affects cardiovascular parameters since the LC plays a central role in controlling autonomic activity through direct and indirect projections to structures involved in cardiovascular regulation [54] (for an overview see [10]). In line with our hypothesis, we observed that heart rate was generally higher for up- versus downregulating pupil size, an effect that increased from session to session and was related to the ability to self-regulate pupil size following pupil-NF training. The effects on HRV indicated by RMSSD values were less clear. Only during fMRI sessions, we found a trend-level effect towards higher HRV in down- as compared to upregulation of pupil size. Since increased HRV has been assumed to be linked to higher activity of the parasympathetic activity alongside decreases in sympathetic activity [68], this tentative result is consistent with the hypothesis that pupil self-regulation modulates cardiovascular markers of arousal [69]. One must keep in mind, though, that the pupil-self regulation period was only 15s. Given this rather short modulation period, our strong effects in heart rate and trend-level effects in HRV are a quite remarkable result. Applying pupil-self regulation for longer periods will allows us to investigate

whether this new intervention can modulate heart rate over longer time scales, supposedly via the LC-NA system that acts on structures in the brainstem and spinal cord involved in autonomic and cardiovascular regulation.

In summary, we provided evidence that our neurofeedback approach allows healthy participants to gain volitional, bidirectional control of their pupil size via mental strategies. Self-regulation of pupil size is associated with systematic modulation of activity in several brainstem nuclei that control the brain's arousal state, including the LC and SN/VTA. We could further show that self-regulation of pupil size modulates (i) cardio-vascular markers of arousal and (ii) psychophysiological and behavioral outcomes measured during an oddball task closely linked to LC activity. Thus, even though pupil-based self-regulation modulates various areas involved in arousal control, our study has revealed convergent evidence suggesting that volitionally controlling pupil size affects LC activity. The introduced pupil-based neurofeedback approach may constitute a completely novel tool to experimentally modulate arousal-regulating neuromodulatory systems in general and LC activity in particular. Considering the strong modulatory effects of the LC-NA system on cognitive function and various behaviors including stress-related responses, pupil-based neurofeedback has an enormous potential to be translated to behavioral and clinical applications across various domains.

Methods

General information

All participants included in the studies were healthy adults, free of medication acting on the central nervous system with no neurological and psychiatric disorders and with normal or corrected-to-normal vision by contact lenses. All participants were asked to abstain from caffeine intake on the day of testing. All experimental protocols were approved by the research ethics committee of the canton of Zurich (KEK-ZH 2018-01078) and conducted in accordance with the declaration of Helsinki. All participants provided written informed prior to study participation and received monetary compensation.

Experiment 1A: Pupil-NF training to learn to volitionally modulate pupil size

Participants

A priori power analyses based on own pilot data of a single training session with 30 trials (unpublished data) aiming for a power level of 80% resulted in a necessary sample size of 31 participants. As we expected training effects of our multi-session approaches, we recruited 28 participants for the NF (24 ± 5 years, 16 females) and 28 participants for the control group (24 ± 5 years; 13 females) of Experiment 1A, exceeding the sample size of other NF studies [34,70,71]. Participants were randomly

assigned to a pupil-NF group and a control group. Developing an eye blink-related strategy instead of a mental strategy (one pupil-NF participant) and a drop-out after the first session due to personal reasons (one control participant) led to a final sample size of n = 27 for the pupil-NF group and n = 27 participants for the control group, respectively.

Pupil-based neurofeedback

Participants sat in a comfortable chair with their chin rested in a chinrest to ensure a stable head position without putting too much strain on the neck of the participants. We kept the height of the chin rest constant across participants and adjusted the height of the chair to accommodate participants [72]. Their eyes were ~65cm away from the eye tracker (Tobii TX300, Tobii Technology AG, Stockholm, Sweden) below the screen (240B7QPJ, resolution: 1680x1050; Philips, Amsterdam, Netherlands) to allow for optimal eye tracking and measurement of pupil size. They were instructed to look at the fixation dot displayed in the center of the screen. Pupil diameter and eye gaze data of both eyes were sampled at 60 Hz. To assure that participants did not use eye movement-related strategies (e.g., vergence movements, squinting), we additionally videorecorded the right eye of the participants and visually inspected these videos. At the start of each session, the eye tracker was calibrated using a 5-point calibration.

In all three training sessions, participants received online, quasi real-time and post-trial feedback on their pupil modulation performance that was based on estimating pupil size of the dominant eye. We accounted for artifacts caused by eye blinks, physiological and measurement-based noise by the following preprocessing steps: (i) rejection of data samples containing physiologically implausible pupil diameter values ranging outside of a pupil size of 1.5 and 9mm – this step also ensured that blinks, recorded with a value of -1, would not be included in feedback shown to participants; (ii) rejection of physiologically implausible pupil size changes larger than a sample-to-sample change of 0.16mm.This previously implemented approach [30,73] is based on specifications of a previous study reporting peak velocity of the pupillary light reflex [74] and converted to our sampling rate. Finally, to ensure a smooth feedback display, the last two collected and processed pupil size samples were averaged and displayed [30]. During the pupil self-regulation phase, pupil size was displayed on the screen by means of a moving circle (see Figure 1C, upper panel) centered around the fixation dot. This moving circle showed pupil size relative to a dashed circle representing the mean pupil size of a 5s baseline phase (i.e., when participants did not self-regulate pupil size). Importantly, the moving circle only indicated pupil size changes in the required direction, e.g., getting larger for upregulation and smaller for downregulation trials. If pupil size didn't change in the required direction, the circle stayed at the size of the baseline circle. The thickness of the circle was adjusted relative to its size so that the numbers of pixels shown on the screen were kept constant throughout the modulation phase. After completion of the self-regulation phase, post-trial performance feedback was displayed. Here, valid

pupil diameter samples were averaged across the modulation phase, the maximum change was extracted and displayed on the screen with average feedback being color-coded: If participants successfully modulated pupil size into the required direction (i.e., pupil size during modulation was larger than baseline pupil size in upregulation trials or smaller than baseline pupil size in downregulation trials), the circle indicating the average change was shown in green (Figure 1C). If pupil size modulation was not successful (i.e., pupil size during modulation was similar to or smaller than the baseline pupil size in the upregulation trials or bigger than baseline pupil size in downregulation trials), the circle was depicted in red. The maximum change was always indicated in black. This intermittent feedback was displayed for 2s.

Throughout the experiment, we ensured that all colors were isoluminant to the grey background (RGB [150 150 150]) by calculating relative luminance as a linear combination of red, green, and blue components based on the formula: $Y = 0.2126\ R + 0.7152\ G + 0.0722\ B$. It follows the idea that blue light contributes the least to perceived luminance while green light contributes the most (https://www.w3.org/Graphics/Color/sRGB). Stimulus presentation throughout the experiment was controlled using MATLAB-based presentation software Psychtoolbox 3.0.17.

All participants underwent three sessions of pupil-NF on separate days within a period of seven days. The pupil-NF training session took place roughly at the same time of the day to keep circadian influences constant. Prior to pupil-NF training on day 1, participants read an instruction sheet explaining the procedures and providing recommended mental strategies derived from previous publications of dilated or constricted pupil size during different mental states and cognitive or emotional tasks ([27,30,38–40]). Participants were informed to rely on these (or similar) mental strategies. Furthermore, we determined the dominant eye of each participant (right eye dominance: n = 22 pupil-NF group; n = 20 control group) since the displayed feedback during training was determined by the data recorded of the dominant eye. Participants were asked to stand ~3m away from a black fixation cross at a white wall, to extend both arms in front of their body along the midline, to form a triangle with their hands, and to look at the fixation cross through this triangle with both eyes open. Then, their right eye was covered. If the fixation cross disappeared through the triangle, then their right eye was recorded as the dominant eye. The same procedure was repeated for the left eye.

In each of the training sessions, three up- and three downregulation blocks were preformed, each consisting of ten trials (30 UP/30 DOWN trials per day; Figure 1B). Each trial (Figure 1C) started with the display of the direction of modulation (either up- or downregulation) in green for 2s on a grey background followed by a baseline phase of 7s. Participants saw a green fixation dot in the center of a screen surrounded by a dashed green circle during baseline. During this baseline phase, participants were instructed to silently count backwards in their head in steps of four to bring participants in a controlled mental state. Then, a 15s modulation phase indicated by the display of an additional solid

green circle (Figure 1C) started in which participants were asked to use mental strategies to up- or downregulate their own pupil size while receiving online pupil-NF. This modulation phase was followed by post-trial performance feedback for 2s. After a break of 5s indicated by the words "short break" in green, a new trial started. After each block, participants could take a short self-determined break before they continued with the next block.

On Training Day 3, participants performed additional no feedback trials following the same overall structure. However, the start of the modulation phase was indicated by a change from an "=" to an "x" in the same color (green) and same position above the fixation dot. No baseline circle was shown and no feedback, neither online nor post-trial performance feedback was provided.

After the pupil-NF sessions, we conducted a debriefing in which participants reported in their own words which mental strategies they used for up- and downregulation, respectively.

Training sessions of the control group

Participants of the control group experienced identical conditions as participants of the pupil-NF group including the same instructions on mental strategies for up- and downregulations and the same amount of training. However, veridical feedback was not provided. To ensure a similar visual input as for the NF group, each participant of the control group saw a version of the visual input from one participant of the NF group resulting in the same proportion of positive/negative performance feedback as in the NF group. However, to prevent the development of illusory correlations, participants were informed that this will occur at a fixed rate. As the pupil NF group, control participants were instructed to look at the fixation dot in the center of the screen and to focus on the mental strategies introduced to them.

Offline data processing and analysis of pupil data

(Pre-)processing of the pupil data was conducted using Matlab R2018a (MathWorks, Inc., Nattick, MA, USA). Recorded pupil size (in mm), gaze data, and video recordings were visually inspected to ensure that participants followed the instructions to look at the fixation dot in the center of the screen during the baseline and modulation phase of the experiment and did not use eye movement related strategies (e.g., vergence movements or squinting). In case of violations during these phases (i.e., squinting/opening eyes systematically, large eye movements/saccades, i.e., deviations > ~16° and ~10° of visual angle on the x- and y-axes, respectively) that can potentially bias the validity of measured pupil size [72], trials were excluded from further analysis (pupil-NF group = 8.5%; control group = 7.4%; $t(52) = -0.8$; $p > .42$). Then, pupil data of both eyes were systematically preprocessed using the guidelines and standardized open source pipeline published by Kret and Sjak-Shie [75]. Invalid pupil diameter samples representing dilation speed outliers and large deviation from trend line pupil size (repeated four times in a multipass approach) were removed using a median absolute deviation (MAD;

multiplier in preprocessing set to 12 [75]) – a robust and outlier resilient data dispersion metric. Further, temporally isolated samples with a maximum width of 50 ms that border a gap larger than 40 ms were removed. Then, we generated mean pupil size time series from the two eyes which were used for all further analyses reported (see [75] on how mean times series can be calculated even with sparsely missing samples of one pupil). The data were resampled with interpolation to 1000Hz and smoothed using a zero-phase low-pass filter with a recommended cutoff frequency of 4 [75]. In a next step, the resulting data were inspected and trials with more than 30% of missing data points across baseline and modulation phases were excluded. Sessions with more than 50% of missing trials of a participant were excluded from further analysis (only n = 1 of the pupil-NF group that developed the eye blink-related strategy). Finally, preprocessed pupil diameter was corrected relative to baseline, by computing mean pupil size of the last 1000ms before the start of the modulation phase of each trial and subtracting this value from each sample of the respective modulation phase, as previously recommended [76].

In a next step, we averaged the baseline-corrected pupil diameter time series during baseline and modulation phases across the UP and DOWN condition of each session (Day 1, Day 2, Day 3, no feedback post) and participant. Furthermore, for each participant and each session, absolute baseline diameter used for trial-based baseline correction (i.e., averaged across 1000ms before modulation onset) was calculated for each condition (i.e., UP and DOWN). To determine whether it's possible to learn to volitionally modulate pupil size, and to determine whether veridical pupil-NF is essential for such successful self-regulation, we calculated a pupil modulation index for all participants of both groups for each session (Day 1, Day 2, Day 3, no feedback post) by subtracting baseline-corrected pupil changes averaged across the 15s modulation phase during DOWN from baseline-corrected pupil changes averages across the 15s modulation phase during UP (i.e., UP – DOWN). Larger modulation indices indicate better condition-specific modulation.

Statistical analyses were performed using IBM SPSS 28 (IBM Corporation, Armonk, NY, USA), R version 4.1.2 (R Core Team), and JASP (version 0.16.2; https://jasp-stats.org/). Since Shapiro-Wilk tests revealed significant deviations from Gaussian distribution of some of the residuals of the pupil modulation index (UP – DOWN) for both groups, we computed robust mixed design ANOVAs based on 20% trimmed means using the R package WRS2 (version 1.1-3; https://cran.r-project.org/web/packages/WRS2/index.html) to compare the pupil modulation indices with the within-subjects factor *session* (Day 1 versus Day 2 versus Day 3 versus Post) and the between-subjects factor *group* (NF versus control). In case of significant effects, we derived post-hoc *p*-values using bootstrap-based functions implemented in the WRS2 package. Finally, we performed statistical analyses to ensure stable pupil size during baseline phases. To this end, we subjected absolute baseline pupil size averaged for each session and condition across participants of each group to a mixed design ANOVA with the within-subjects factors *session* (Day 1 versus Day 2 versus Day 3 versus Post) and *condition*

(UP versus DOWN) and the between-subjects factor *group* (pupil-NF versus control). In case of significant effects, we derived post-hoc *p*-values sequential Bonferroni-corrected for multiple comparisons [41]. Additionally, we ran a Bayesian ANOVA using the same factors to be able to evaluate whether there is evidence for the null hypothesis (i.e., no difference in baseline pupil size between conditions in neither session and/or group).

Experiment 1B: Replication of Experiment 1A

Participants

We recruited an independent cohort of 26 participants (18 females, 26 ± 7 years). Technical issues with the eye tracker led to an interruption of training and exclusion of one participant (remaining n = 25).

Pupil-based Neurofeedback

The paradigm used was identical to Experiment 1A with two implemented changes: (i) we added a no feedback session *prior* to training (Pre) in which participants only used instructed mental strategies without receiving any feedback; (ii) online feedback on training Day 3 was removed and participants only received post-trial performance feedback. As during no feedback trials, the baseline phase was indicated by a "=" above the fixation dot on the screen, changing to an "x" as soon as the modulation phase started. Trial timing and instructions remained the same as in Experiment 1A**.**

Cardiovascular measurements

For 15 participants (11 females, 25 ± 6 years), we additionally recorded cardiac data by a Biopac MP 160 system and the accompanying AcqKnowledge software (Biopac Systems Inc., USA). ECG was recorded continuously and sampled at 1000Hz from two electrodes, one attached to the left lower rib and under the right collarbone. An electrode attached to the left collarbone was used as a reference. All physiological data was recorded and stored on a PC for off-line analysis. We also recorded continuous respiration data by means of a breathing belt (Biopac Systems Inc., USA) that was affixed to the participant's chest, however, respiratory data is not reported here.

Pupil data (pre-)processing and analysis

Experiment 1B in which we implemented a no feedback session Pre as well as Post pupil-NF training allowed us to directly compare self-modulation performance without feedback effects in the same group of participants. We excluded 5.4% of all trials due to violations during baseline and modulation phases (i.e., squinting/opening of the eye in a systematic way, large eye movements/saccades).

Baseline-corrected pupil size time series during self-regulation were calculated as described in Experiment 1A. We statistically compared these time series Pre and Post pupil-NF training by subjecting the data to a two-way repeated measures ANOVA with the within-subjects factors *condition* (UP versus DOWN) and *session* (Pre versus Post pupil-NF training) using the MATLAB-based SPM1D toolbox for one-dimensional data (SPM1D M.0.4.8; https://spm1d.org/). SPM1D uses random field theory to make statistical inferences at the continuum level regarding sets of one-dimensional measurements. It is based on the idea to quantify the probability that smooth, random one-dimensional continua would produce a test statistic continuum whose maximum exceeds a particular test statistic value and has been previously used to analyze one-dimensional kinematic, biomechanical or force trajectories [77,78]. In case of significant effects, post-hoc tests implemented in the SPM1D software were used which are corrected for multiple comparisons using Bonferroni corrections.

Cardiac data (pre-)processing and analyses

ECG R peaks were detected automatically and, if necessary, manually corrected using the MATLAB-based toolbox PhysioZoo [79]. Data segments consisting of non-detectable peaks or poor quality were excluded from further analyses. Resulting R-R intervals for which both R peaks were occurring in the modulation phase of the pupil-NF training were extracted and further processed in MATLAB (R2021a). Data of one participant was disrupted and excluded since it was not possible to detect R peaks for no feedback trials prior to training and training session D1. Missing data was replaced by the group mean to not further reduce statistical power. Heart rate, reflecting cardiovascular dynamics controlled by an interplay between the sympathetic and parasympathetic nervous system, was calculated by dividing 60 through the respective R-R intervals of the modulation phase. Furthermore, we computed the RMSSD calculated based on R-R-intervals and representing a beat-to-beat measure of HRV in the time domain that is assumed to give meaningful insights about parasympathetic activity of the ANS for time windows as short as 10 s [51,52]. Heart rate and RMSSD calculated for each modulation phase was averaged across the respective up- and downregulation condition of each training session.

To investigate whether pupil self-modulation systematically modulates cardiovascular parameters, we subjected heart rate averages for each condition (UP versus DOWN) and training session (no feedback pre, Day 1, Day 2, Day 3, no feedback post) to a two-way repeated measures ANOVA. Since our HRV measure, RMSSD, significantly deviated from normal distribution (Shapiro-Wilk tests, all $p < .01$), we calculated differences scores in RMSSD between up- and downregulation trials (DOWN-UP) for each session (no feedback pre, Day 1, Day 2, Day 3, no feedback post). Positive values indicated larger HRV in DOWN than UP trials and negative values indicated larger HRV in UP than DOWN trials. These difference scores were subjected to a robust ANOVA using the WRS2 package of R. In case our analyses yielded significant effects, post-hoc tests were conducted. To determine whether potential

differences in cardiac dynamics between UP and DOWN trials were associated with volitional pupil modulation performance prior to training, we calculated Spearman correlation coefficients between the pupil modulation index (i.e., UP – DOWN) and heart rate (UP – DOWN) or RMSSD (DOWN – UP). Reported statistical analyses were two-tailed tests and corrected for multiple comparisons using sequential Bonferroni correction [41] or Hochberg's approach implemented in the WRS2 package of R.

Experiment 2: Pupil-NF combined with simultaneous fMRI

Participants

For Experiment 2, we re-recruited 25 participants (17 females; age 25 ± 5 years) from the pupil-NF groups of Experiments 1A, B, a sample size that is bigger or comparable to those in previous fMRI studies showing significant correlations between pupil measures and brain activity [17,19,20]. Additional exclusion criteria were: (i) metal implants and/or fragments in the body, (ii) suffering from claustrophobia, and (iii) pregnancy at the time of the experiment. In case the last session of pupil-NF took place more than ten days before the scheduled fMRI session, participants received one session of re-training combining 40 trials of online feedback (i.e., 20 trials UP and DOWN, respectively) and 20 trials of post-trial feedback (i.e., 10 trials UP and DOWN, respectively) in the week before the first fMRI session. This re-training was included to assure that participants were able to modulate their own pupil size still successfully. Data of this re-training is not reported here.

Data acquisition

All volunteers participated in two fMRI sessions, one using an optimized brainstem imaging sequence, the other acquiring whole brain data (order of sessions was counterbalanced across volunteers). (f)MRI data was acquired on a 3T Philips Ingenia system using a 32-channel head coil. Anatomical images of the brain used for anatomical co-registration were acquired using a T1-weighted sequence (MPRAGE; 160 sagittal slices, TR/TE = 9.3/4.4 ms, voxel size = 0.7 mm^3, matrix size = 240 x 240, flip angle = 8°, FOV = 240 mm (AP) x 240 mm (RL) x 160 mm (FH)). A turbo spin echo (TSE) neuromelanin-sensitive structural scan was acquired for delineation of the LC not reported here which was oriented perpendicular to the floor of the fourth ventricle (20 slices, 1.5mm, no gaps, in-plane resolution: 0.7 x 0.88 mm (reconstructed at: 0.35 x 0.35 mm), TR = 500 ms, TE = 10 ms, flip angle = 90˚, covering the brainstem only). During the brainstem session, echo-planar imaging (EPI) scans were acquired in 39 sagittal slices (thickness: 1.8 mm, no gaps) aligned parallel to the floor of the fourth ventricle along the brainstem with the following parameters: TR = 2.5 s, TE = 26 ms, flip angle = 82°, SENSE acceleration factor = 2.1, in-plane resolution of 1.8 x 1.8 mm, and 240 volumes per run. Additionally, a whole-brain single volume EPI image was acquired to improve co-registration between

fMRI data and the anatomical MRI scans (identical sequence parameters to the brainstem fMRI sequence).

During the whole brain session, EPI images were acquired in 40 slices (thickness: 2.7 mm) with the following parameters: TR = 2.5 s, TE = 30 ms, flip angle = 85°, FOV = 223 mm (AP) x 223 mm (RL) x 116 mm (FH), Sense factor 2. Images were acquired at an in-plane resolution of 2.7 x 2.7 mm and were reconstructed at a resolution of 1.74 x 1.74 mm. In each session, we acquired 240 volumes per run. For the first seven subjects, we used an EPI sequence with slightly different details (i.e., especially higher spatial resolution to be able to see brainstem activity in small nuclei; non-isometric: 36 slices (thickness: 3mm, no gaps, TR = 2.5, TE = 30, flip angle 82°, FOV = 210 mm (AP) x 210 mm (RL) x 108 mm (FH), Sense factor 2.2, in plane resolution of 2 x 2 mm reconstructed at a resolution of 1.64 x 1.64 mm), however, due to the strong distortion observed in one excluded participants, we decided to adjust the sequence.

During both MRI sessions, cardiac cycle and respiratory activity were recorded with a sampling rate of 496 Hz with a pulse oximeter attached to the left index finger and a chest belt, respectively (Invivo; Phillips Medical Systems, Orlando, Florida). These data were used for physiological noise removal as well as for calculating cardiovascular parameters.

Concurrently with fMRI recordings, participants' eye gaze and pupil size of the left eye were tracked at 1000 Hz using an EyeLink 1000 Plus Long Range Mount (SR Research, Ottawa, Ontario, Canada) that was placed at the end of the scanner bore on an aluminum stand and a first-surface mirror attached to the head coil. At the beginning of each fMRI run, the eye tracker was calibrated using 5-point calibration implemented in the software of the device. Pupil diameter was converted from arbitrary units to mm using the following formula: diameter (mm) = diameter (a.u.) / 1372. The conversion factor was estimated as described in [72,80].

fMRI Paradigm

The pupil-NF paradigm during fMRI was similar to our training paradigm. Participants were instructed to maintain gaze on the green fixation dot presented in the center of the screen. Each trial began with a baseline measurement (7.5s) followed by an UP or DOWN phase (15s; without online feedback) during which participants were asked to apply the acquired respective mental strategy. Participants only received post-trial performance feedback (2.5s). After a short break (randomly jittered between 6-9s), the next trial started. Online processing and calculation of the feedback displayed to participants was performed as in Experiment 1A. The conditions were blocked within each of four fMRI runs, that is four trials of upregulation followed by four trials of DOWN (or vice versa). At the beginning of each block, it was indicated on the screen, whether this block contains UP or DOWN trials. After each block, a break of 10 s was added to allow participants to take short breaks in between. Each of the runs

comprised two blocks of each condition, leading to 32 trials per condition. The order of conditions was counterbalanced across runs. Further, we counterbalanced across participants with which conditions they started the respective fMRI session.

Processing and analysis of pupil data

Pupil data (pre-)processing was conducted as described for Experiment 1A leading to baseline-corrected pupil diameter time series for UP and DOWN trials for each fMRI session. Similar to Experiment 1, absolute baseline pupil diameter was compared by means of a repeated measures ANOVA with the within-subjects factor *session* (brainstem versus whole-brain) and *condition* (UP versus DOWN) and Bayesian repeated measures ANOVAs.

Finally, preprocessed, absolute pupil diameter data throughout the whole experiment was downsampled to match the fMRI volume acquisition by averaging all data within a given TR. Missing pupil diameter were linearly interpolated across adjacent epochs resulting in a pupil diameter vector for each fMRI run.

Processing and analysis of cardiac data

Heart (pulse) rate concurrently measured during fMRI was (pre-)processed as described for Experiment 1B resulting in heart rate and RMSSD averages for each condition (UP and DOWN) and session (brainstem and whole-brain). Due to technical issues (whole-brain session of 1 participant), poor data quality (brainstem session of 1 participant), the final n for heart rate and RMSSD analyses was 24 for each fMRI session. Heart rate data was subjected to a repeated measures ANOVA with the within-subjects factor condition (UP versus DOWN) and fMRI session (brainstem vs. whole-brain). Since RMSSD values did significantly deviate from normal distribution (revealed by Shapiro-Wilk tests $p < .05$), we compared UP and DOWN trials for each fMRI session by means of a Wilcoxon signed rank test implemented in SPSS. Finally, as for the no feedback session prior to pupil-NF training, we calculated Spearman correlation coefficients between the pupil modulation index (UP - DOWN) and differences in heart rate (UP – DOWN) or RMSSD (DOWN – UP) following training during fMRI (averaged across the two sessions). All statistical analyses were computed using SPSS and corrected for multiple comparison using sequential Bonferroni correction.

MRI analysis

MRI data was analyzed using tools from FSL version 6.0 (http://fsl.fmrib. ox.ac.uk/fsl), MATLAB R2018b, and FreeSurfer version 6.0 (https://surfer.nmr.mgh.harvard.edu/).

Pre-processing of brainstem data

Since brainstem fMRI signal is highly susceptible to corruption from physiological sources of noise, we used a rigorous pre-processing pipeline implementing suggestions from previously published research [17,23,81] including the following steps: brain extraction using FSL's automated brain extraction tool (BET; [82]), motion correction using Linear Image Registration Tool (MCFLIRT;[83]), spatial smoothing using a 3mm full width-at-half-maximum (FWHM) Gaussian kernel, and a 90s high-pass temporal filter as implemented via FSL's Expert Analysis Tool (FEAT;[84]). Additionally, each functional run showing excessive motion with an absolute mean displacement greater than 1 mm (i.e., ~half a voxel size) was excluded (3 runs for 1 participant which led to exclusion from further analyses). To further de-noise the data, we performed Independent Component Analysis (ICA) and FLS's Physiological Noise Modelling (PNM;[85]), an extended version of RETROICOR [86]). ICA denoising was performed using FSL's Multivariate Exploratory Linear Optimized Decomposition interface (MELODIC, [87]), which allows for decomposition of the fMRI data into spatial and temporal components. The resulting components were visually inspected and classified as "Noise" or "Signal" by two independent researchers using published guidelines [88]. Once labelled, the temporal information of the noise components was extracted and ICA noise regressor lists were generated for later implementation in our general linear model (GLM). For PNM, cardiac and respiratory phases were assigned to each volume in the concatenated EPI image time series separately for each slice. Our complete physiological noise regression model included 34 regressors: 4th order harmonics to capture the cardiac cycle, 4th order harmonics to capture the respiratory cycle, 2nd order harmonics to capture the interaction between cardiac and respiratory cycles, 2nd order harmonics to capture the interaction between respiratory and cardiac cycles, one regressor to capture heart rate, and one regressor to capture respiration volume. Subsequently, we visually inspected the waveforms to assure that the peak of each respiratory and cardiac cycle was correctly marked and adjusted if necessary. The resulting voxelwise confound lists were later added to our GLM. Note that physiological data acquisition was corrupted for one participant. Therefore, this data was analyzed without the PNM regressors modelled. Image co-registration from functional to Montreal Neurological Institute (MNI) standard space was performed by (i) aligning the functional images of each run to each subject's whole-brain EPI image, using the Linear Image Registration Tool (FLIRT) employing a mutual information cost function and six degrees of freedom [89]; (ii) registering whole-brain EPIs to structural T1-weighted images, using a mutual information cost function six degrees of freedom, and then optimised using boundary-based registration (BBR;[90]); (iii) co-registering structural images to the MNI152 ($2mm^3$) template via the nonlinear registration tool (FNIRT) using 12 degrees of freedom [91], and applying the resulting warp fields to the functional images. Each co-registration step was visually inspected using Freeview (FreeSurfer) to ensure exact alignment with an emphasis placed on the pons regions surrounding the LC. Unfortunately, 3 out of 25 participants needed to be excluded from brainstem fMRI analyses due

to heavy distortions (including brainstem regions; 1 participant), excessive motion (1 participant, see above), and periods of sleep during the measurement (1 participant).

Pre-processing of whole-brain data

We used a similar pre-processing pipeline as described above, however, with the following differences: Spatial smoothing was applied using a 5mm full width-at-half-maximum (FWHM) Gaussian kernel. Functional EPI-images were aligned to structural T1-weighted images using linear registration (FLIRT). Structural images were aligned to the MNI standard space using nonlinear registration (FNIRT), and the resulting warp fields applied to the functional images. Data from 1 out of 25 participants showed very strong distortions in temporal and frontal regions and was excluded from further analysis. Also, we needed to exclude 1 functional run for 3 participants and 2 functional runs for 1 participant due to excessive motion with an absolute mean displacement greater than half a voxel size (i.e., 1.4 mm). We did not apply PNM to the data and, as recommended by FSL (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FEAT/UserGuide) ICA was only performed (n = 9) if residual effects of motion were still left in the data even after using MCFLIRT for motion correction.

fMRI analyses

Time-series statistical analysis were performed using FEAT with FMRIB's Improved Linear Model (FILM). Two equivalent GLMs were estimated for each session (i.e., for the whole-brain session and the brainstem session).

First, we directly contrasted brain activity during up- versus downregulation periods during whole-brain scans. Therefore, "UP" and "DOWN", were modelled as main regressors of interest for the complete modulation phase of 15 s. Instruction, baseline, and intermittent feedback periods were modelled as separate regressors. All regressors were convolved with a double gamma HRF and its first temporal derivative. White matter (WM) and cerebrospinal fluid (CSF) time-series, motion parameters and ICA noise component time-series (if applied), respectively, were added as nuisance regressors. Second, we investigated which brain areas would change their activity in close association with pupil diameter. Therefore, pupil diameter was entered as a regressor of interest, together with the same nuisance regressors as described above.

For the brainstem session, we defined GLMs with identical regressors that were, however, convolved with the default optimal basis sets (i.e., the canonical hemodynamic response function (HRF) and its temporal and dispersion derivatives) using FMRIB's Linear Optimal Basis Sets toolkit [92]. This convolution was chosen to account for the possibility that brainstem BOLD responses such as in the LC are not well-modelled solely by the canonical HRF [93]. However, to avoid overestimating statistical effects, only the canonical HRF regression parameter estimates were used for subsequent

higher-level analyses. Additionally, the PNM voxelwise confound lists and the time series of ICA noise components lists were used as nuisance regressors.

All described GLMs were computed separately for each run of each participant and then averaged across runs for each participant in second level analyses using FMRIB's Local Analysis of Fixed Effects [94]. To determine significant effects at the group level, a third level mixed-effects analyses was performed. Group Z-statistic images were thresholded at $Z > 3.1$ for whole-brain analyses and family-wise-error corrected (FWE) at the cluster level. In line with a previous publication [23], we report results for the brainstem analysis at an uncorrected $p < 0.05$, taking into account the limited signal-to-noise ratio for brainstem responses and the small size of the LC in combination with our strict pre-processing pipeline. Here, we implemented a minimum cluster size of 10 voxels, derived from the smallest mask of a recently published brainstem atlas covering all important brainstem nuclei involved in arousal control [42–48]. To visualize brainstem activity, we created a mask covering the brainstem (derived from the Harvard-Oxford subcortical atlas; thresholded at 0.5 and binarized using FSL's image calculator fslmaths) and the NBM (Ch4 cell group derived from the JuBrain/SPM Anatomy Toolbox; [49]), showing Z-statistic images within these predefined regions.

Regions of Interest (ROI) analysis

We conducted a series of ROI analyses, to test our hypothesis that self-regulation of pupil size is linked to activity changes in the LC and other neuromodulatory, arousal-regulating centres in the brain. Our principal region of interest was (i) the LC, however, given the close interaction with other brainstem areas and neuromodulatory systems involved in pupil control, we defined secondary ROIs, namely (ii) the cholinergic NBM, (ii) the dopaminergic SN/VTA, (iii) the serotonergic DRN, and (iv) the SC – a specialized motor nucleus in the midbrain implicated in the pupil orientation response – as a control region. Probabilistic anatomical atlases were used to define the location of the brainstem ROIs [42–48] and the NBM (Ch4 cell group; [49]; see Figure 3F) in MNI space. After careful visual inspection, we decided to threshold the SC and DRN mask at 0.1, and the SN/VTA-mask at 0.3. The masks for all ROIs were binarized. In a next step, we extracted the average mean signal intensity (Z-values) within each ROI from the second-level fixed effects analysis conducted for each participant for each GLM (i.e., for UP versus rest and DOWN versus rest, and pupil diameter vector used as regressor). Due to deviation from normal distribution for the NBM (Shapiro-Wilk tests; $p < .05$), we used Wilcoxon signed rank tests to test for a significant difference between Z-values for UP and DOWN periods within each ROI, as well as for a significant correlation between absolute pupil diameter and BOLD activity within each ROI (i.e., > 0). All analyses were corrected for multiple comparisons using sequential Bonferroni correction.

Experiment 3: Pupil-NF combined with an auditory oddball task

Participants

Experiment 3 involved 22 participants (15 females; 26 ± 6 years) of the 25 participants re-recruited for the fMRI experiment. Unfortunately, 3 participants dropped out because of personal reasons. Participants received one session of re-training combining 40 trials of online feedback (i.e., 20 trials UP and DOWN, respectively) and 20 trials of post-trial feedback (i.e., 10 trials UP and 10 DOWN, respectively) within 7 days prior to Experiment 3, in case the last session of pupil-NF took place more than ten days before the scheduled pupil-NF oddball session. This re-training was included to assure that participants were able to modulate their own pupil size successfully. Data of this re-training is not reported here.

Paradigm: Pupil-NF with an integrated auditory oddball task

The auditory oddball task during pupil-NF consisted of 186 target (~20%) and 750 standard (~80%) tones (440 and 660 Hz, pitch for target was counterbalanced across participants) delivered for 100 ms via headphones at approximately 60dB (Panasonic RP-HJE125, Panasonic Marketing Europe GmbH, Wiesbaden, Germany), and was embedded in our pupil-NF paradigm during which participants were volitionally up- or downregulating their own pupil size via acquired mental strategies. In addition to these two self-regulation conditions, we added a third control condition in which participants were asked to count back in steps of seven instead of self-regulating pupil size. To ensure that participants stick to this task, they were occasionally prompted to enter the final number (after every 6th counting trial). This condition was added to control for effort-effects related to pupil self-regulation and to check whether oddball responses differ in self-regulated as compared to more natural fluctuations in pupil size. At the beginning of the task, participants were reminded to focus on their mental strategies and to pay attention to the tones at the same time.

Each pupil-NF-oddball trial began with a short instruction (2s) indicating the required pupil self-regulation direction (i.e., UP or DOWN) or the counting control condition followed by a baseline measurement of 4s in which participants were asked to silently count backwards in steps of seven. Then, participants were asked to apply the acquired mental strategy to up- or downregulate pupil size for 18s or to continue counting (i.e., control). During this self-regulation or counting phase, eight tones (one or two targets and seven or six standards) were played, and participants were asked to respond to targets but not to standards by pressing a key as quickly as possible with the index finger of their preferred hand. The first tone which was always a standard in each self-regulation or counting phase was played after the first jitter phase (1.8 – 2.2 s after the baseline phase) to ensure that participants already reached some modulation of pupil size before the first target appeared. The inter-stimulus interval (ISI) between tones was randomly jittered between 1.8 – 2.2 s. Between two targets, there

were at least two standards presented leading to a minimum ISI of 5.4 s between two targets allowing the target-evoked pupil dilation response to return to baseline between targets. After each trial, participants received post-trial feedback on average and maximum pupil diameter change for 2s. After a short break of 2s, the next trial started. The calculation of feedback was similar as described in Experiment 1A, except that only the last two seconds of the baseline phase were considered due to the shortened baseline phase of 4s. For counting trials without feedback phases, counting was followed by a 4s break. Instructions and indication of task phases on the screen were displayed as in Experiment 1B on Day 3 (Figure 6A).

The pupil-NF conditions were blocked, each block containing 13 trials of UP, DOWN, and control, respectively. The number and order of tones was the same for each pupil-NF condition. After each block, participants could take a self-paced break. In total, the task consisted of nine blocks leading to 117 trials pupil-NF trials in total. The order of blocks was pseudorandomized in the sense that each condition needed to be presented once (or twice) before another condition was presented for the 2nd (or 3rd) time.

Physiological data acquisition

Eye gaze, pupil diameter, cardiac and respiratory data was recorded throughout the task as described in Experiment 1A, B. Additionally, we acquired electroencephalography (EEG) data using 64 Ag/AgCl actiCAP active surface electrodes, an actiCHamp Plus amplifier and the Brain Vision Recorder proprietary software (Brain Products, Munich, Germany). However, cardiac, respiratory and EEG data are not reported here.

Data (pre-)processing and statistical analyses

Pupil-NF: Self-regulation of pupil size under dual task conditions

Pupil data (pre-)processing was conducted as described for Experiment 1A. We had to exclude 2 participants from further analyses leading to a final n = 20 for pupil data analyses: one participant showed a very high blink rate during the oddball with more than 30% of missing samples during baseline and self-regulation/counting phases for more than 50% of the trials. A second participant showed a similar pattern with more than 30% of missing samples in 35% of the trials, mainly in the control condition (only 20% of the trials were usable after pre-processing). Combined with low behavioral performance (correct responses to targets deviated more than 3 SD from the group mean), we decided to exclude this participant from pupil and behavioral data analyses. For each of the remaining participants, we derived *abolute* and *baseline-corrected* pupil diameter time series averaged for UP, DOWN or counting trials throughout the baseline and self-regulation/counting phase of the pupil-NF oddball task. Both absolute and baseline-corrected pupil diameter time series were extracted

since we expected to observe target-evoked pupil dilation responses that depend in size on absolute pupil size prior to tone onset. Baseline correction was applied as described above, choosing a baseline of 1s prior to self-regulation and counting, respectively.

For statistical analyses of self-regulated pupil size, we averaged preprocessed, baseline-corrected pupil diameter time series for each condition across the entire self-regulation or counting phase to test whether participants were able to successfully self-regulate pupil size even under challenging dual-task conditions. Since these data were deviating from normal distribution (Shapiro Wilk test; $p < .05$), we subjected the data to a robust repeated measures ANOVA implementing 20% trimmed means with the factor *condition* (UP versus DOWN versus control; R package WRS2). In case of a significant main effect, we computed the corresponding post hoc tests included in this R package with implemented FWE correction. Next, we averaged absolute pupil diameter samples across the last second of the baseline phase and subjected it to a repeated measures ANOVA with the within-subjects factor *condition* (UP versus DOWN versus control) to compare absolute baseline pupil diameter between the three conditions. In case of a significant effect, post-hoc tests were conducted using the sequential Bonferroni correction for multiple comparisons. Additionally, we ran an analogous Bayesian repeated measures ANOVA using JASP to evaluate whether there is evidence for the null hypothesis (i.e., no baseline differences between conditions).

Pupillary responses to presented tones during the oddball task: Pre-tone baseline pupil diameter and pupil dilation responses

We extracted 3.5-s epochs around each standard or target tones from -0.5 to +3s relative to tone onset. Pre-tone pupil diameter was calculated by averaging 500ms of pupil diameter data preceding tone presentation. The pupil time series was then (i) normalized to this pre-tone pupil diameter baseline and (ii) averaged for each stimulus type (i.e., standard versus target) and condition (i.e., UP versus DOWN versus control).

To particularly compare *target*-evoked pupil dilation responses between conditions, we subjected the baseline-corrected pupil dilation response time series to targets grand-averaged within each condition to a repeated-measures ANOVA with the within-subjects factor *condition* (UP versus DOWN versus control) using the SPM1D toolbox. This time series data analysis allowed us to compare both peak dilation towards targets and the shape of the dilation response statistically. The same analysis was repeated for pupil dilation responses to standard tones as a control analysis. In case of a significant main effects, post-hoc tests implemented in the SPM1D software corrected for multiple comparisons using Bonferroni correction were used.

Behavioral data

Throughout the experiment, we recorded the correctness of responses and reaction times to tones. Responses to standard tones (i.e., false alarms) and responses for which reaction times exceeded two SD below or above the mean of the respective participant were excluded from further reaction time analyses. Lastly, we calculated the percentage of hits (i.e., responses to targets) and false alarms (i.e., responses to standards) and obtained a percentage of correct responses by subtracting these two measures from each other [67].

To investigate whether self-regulation of pupil size modulates behavioral task performance on the oddball task, we subjected individual reaction times to target tones averaged across trials of each condition to a repeated measures ANOVA with the within-subjects factor *condition* (UP versus DOWN versus control). Next, we examined whether not only speed per se but also performance variability indicated by the standard deviation (SD) of reaction times may be modulated by self-regulated pupil size. We subjected the SD of reaction times averaged across the respective conditions to a repeated measures ANOVA with the within-subjects factor *condition* (UP versus DOWN versus control). In case of a significant effect, we conducted post-hoc tests controlled for multiple comparisons by means of sequential Bonferroni corrections.

Correlational analyses between pupil and behavioral data

Finally, we computed repeated measures correlation coefficients between the derived single-trial pre-tone absolute baseline pupil diameter (averaged 500ms before target onset), baseline-corrected target-evoked pupil dilation responses, and reaction times to targets of all participants using the R package rmcorr [95].

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