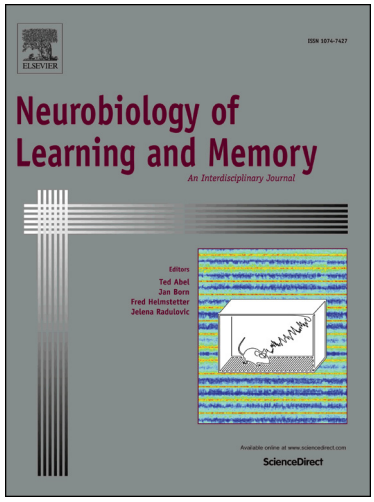

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PII: S1074-7427(17)30069-2
DOI: http://dx.doi.org/10.1016/j.nlm.2017.05.006
Reference: YNLME 6684

To appear in: *Neurobiology of Learning and Memory*

Received Date: 13 January 2017
Revised Date: 22 April 2017
Accepted Date: 7 May 2017

Please cite this article as: Klarer, M., Weber-Stadlbauer, U., Arnold, M., Langhans, W., Meyer, U., Cognitive Effects of Subdiaphragmatic Vagal Deafferentation in Rats, *Neurobiology of Learning and Memory* (2017), doi: http://dx.doi.org/10.1016/j.nlm.2017.05.006

Cognitive Effects of Subdiaphragmatic Vagal Deafferentation in Rats

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Running title: Abdominal vagal afferent signaling and cognition

Key words: Cognitive flexibility; vagus nerve; recognition memory; reversal learning; working memory.

Abstract

Vagal afferents are a crucial neuronal component of the gut-brain axis and mediate the information flow from the viscera to the central nervous system. Based on the findings provided by experiments involving vagus nerve stimulation, it has been suggested that vagal afferent signaling may influence various cognitive functions such as recognition memory and cognitive flexibility. Here, we examined this hypothesis using a rat model of subdiaphragmatic vagal deafferentation (SDA), the most complete and selective abdominal vagal deafferentation method existing to date. We found that SDA did not affect working memory in a nonspatial alternation task, nor did it influence short-, intermediate-, and long-term object recognition memory. SDA did also not affect the acquisition of positively reinforced left-right discrimination learning, but it facilitated the subsequent reversal left-right discrimination learning. The SDA-induced effects on reversal learning emerged in the absence of concomitant changes in motivation towards the positive reinforcer, indicating selective effects on cognitive flexibility. Taken together, these findings suggest that the relative contribution of vagal afferent signaling to cognitive functions is limited. At the same time, our study demonstrates that cognitive flexibility, at least in the domains of positively reinforced learning, is subjected to visceral modulation through abdominal vagal afferents.

Highlights

- Abdominal vagal deafferentation facilitates positively motivated reversal learning.
- Abdominal vagal deafferentation does not affect object recognition memory.
- Abdominal vagal deafferentation does not affect working memory.

1. Introduction[1]

The central nervous system (CNS) constantly receives signals from the viscera via neural and endocrine routes (Mayer 2011). These signals are part of an interoceptive pathway that can directly and indirectly influence various brain functions such as perception, emotion, and reward- or goal-directed behavior (Critchley and Harrison 2013). The vagus nerve is one of the key neuronal elements mediating visceral influences on the brain (Berthoud and Neuhuber 2000). It is the tenth cranial nerve and consists of 80% afferent sensory nerve fibers (Agostoni et al. 1957; Berthoud and Neuhuber 2000). Vagal afferent neurons synapse bilaterally on the nucleus tractus solitarii (NTS), from where visceral signals are conveyed to various brain stem nuclei and forebrain structures (Barnes et al. 2003; Berthoud and Neuhuber 2000; Cechetto 1987; Childs et al. 2015; Khalsa et al. 2009).

Disruption of vagal afferent signaling has been associated with a failure to convey gut-derived signals from the viscera to the CNS (Cryan and Dinan 2012), which in turn may contribute to changes in mood and affect (George et al. 2003; Groves and Brown 2005; Klarer et al. 2014). Based on the findings provided by experiments involving vagus nerve stimulation (VNS), it has been suggested that vagal afferents may be an endogenous mediator of certain cognitive functions (Vonck et al. 2014). For example, VNS has been shown to enhance the retention of inhibitory avoidance memory (Clark et al. 1995; 1998) and to facilitate the extinction of conditioned fear (Pena et al. 2014; 2013) in rats. Furthermore, VNS has been found to improve word-recognition memory (Clark et al. 1999) and response selection during action cascading processes (Steenbergen et al. 2015) in human subjects. These cognitive effects have been attributed to modulation of central noradrenergic (NA) and γ-aminobutyric acid (GABA) systems (Beste et al. 2016;

Abbreviations: CCK, cholecystokinin; CNS, central nervous system; CS, to-be-conditioned stimulus; GABA, γ-aminobutyric acid; HPC, hippocampus; ITI, inter-trial interval; LC, locus coeruleus; (m)PFC, medial prefrontal cortex; NA, noradrenalin; NORT, novel object recognition test; NTS, nucleus tractus solitarii; OFC, orbitofrontal cortex; SDA, subdiaphragmatic vagal deafferentation; TVX, total subdiaphragmatic vagotomy; US, unconditioned stimulus; VNS, vagus nerve stimulation

Manta et al. 2013; Steenbergen et al. 2015), as well as to neuronal adaptations within limbic and cortical brain areas such as amygdala, hippocampus (HPC), and prefrontal cortex (PFC) (Pena et al. 2014; Zuo et al. 2007).

Some of the reported cognitive effects of VNS, however, remain controversial (Boon et al. 2006; Ogbonnaya and Kaliaperumal 2013; Vonck et al. 2014), which in turn may undermine current interpretations surrounding the involvement of vagal afferents in cognition. Therefore, we sought to examine the contribution of abdominal vagal afferent signaling to cognitive functions using a rat model of subdiaphragmatic vagal deafferentation (SDA). SDA leads to complete disconnection of all abdominal vagal afferents whilst sparing half of the vagal efferents (Arnold et al. 2006; Norgren and Smith 1994). To date, it is the most complete and selective vagal deafferentation method for all abdominal visceral fibers and critically differs from total subdiaphragmatic vagotomy (TVX) models, the latter of which leads to a disconnection of both the afferent and efferent fibers of the vagus nerve below the diaphragm (Bercik et al. 2011; Bravo et al. 2011). Unlike TVX (Kraly et al. 1986), SDA allows for a discrimination of the relative functional contribution of vagal afferents versus efferents in the absence of severe side effects such as disturbances in gastrointestinal motility and secretion, hypophagia and subsequent body weight loss (Arnold et al. 2006; Azari et al. 2014; Klarer et al. 2014). Based on the reported effects of VNS on working memory (Beste et al. 2016), recognition memory (Clark et al. 1999), and cognitive flexibility (Ghacibeh et al. 2006a), we compared the performance of SDA and Sham-operated rats in these cognitive domains.

2. Materials and Methods

2.1. Animals

Adult (280-320 g) male Sprague Dawley (Crl:CD) rats (Charles River, Sulzfeld, Germany) were used throughout the study. Animals were group-housed (2-4 animals per cage) in acrylic stainless-steel grid-floor cages (Type IV, 595mm × 380mm × 200mm) and kept

under a reversed light-dark cycle (lights on from 20h00 to 08h00) at 22 ± 2 °C and 55-60% humidity. The animals had ad libitum access to water and standard chow (Kliba 3436, Provimi Kliba, Kaiseraugst, Switzerland), unless otherwise specified. Prior to surgery, the animals were allowed to acclimatize to the new animal holding facility for 3 weeks, during which they were handled on a daily basis in order to habituate them to the experimenter. All procedures were approved by the Cantonal Veterinarian's Office of Zurich. All efforts were made to minimize the number of animals used as well as their suffering.

2.2. SDA and Sham surgeries

Five days before surgery, all rats, independent of their group designation, were nursed with special diets, alternately consisting of unsweetened condensed milk (Migros, Switzerland) and wet mash (unsweetened condensed milk mixed with powdered ground chow, Kliba 3433; Provimi Kliba Nafag) to avoid excessive weight loss post-surgery. This feeding regimen was continued until 5 days post-surgery, when the animals were re-adapted to standard rodent ground chow (Kliba 3436; Provimi Kliba Nafag) and given access to ad libitum chow thereafter.

The SDA and Sham surgeries were performed as established and explained in detail before (Arnold et al. 2006; Klarer et al. 2014; Labouesse et al. 2012). In brief, SDA consisted of a left-side intracranial vagal rhizotomy and a transection of the second, dorsally located (right) subdiaphragmatic trunk of the vagus nerve. Sham surgery consisted of similarly exposing both the vagal rootlets and dorsal subdiaphragmatic vagus, but without manipulating them. The SDA results in a complete disconnection of abdominal vagal afferents, while half of the abdominal vagal efferents are spared (see Figure 1***A***). Above the diaphragm, all vagal efferent fibers are left intact and some functional vagal afferents remain. Complete and uniquely vagal deafferentation at the cervical level through bilateral rhizotomy causes severe breathing complications after surgery, and as a consequence of this, rats will die within 48 h post-surgery (M. Arnold; unpublished observations). Given these limitations of bilateral cervical rhizotomy, the SDA

procedure is still the most complete and selective abdominal vagal deafferentation method existing to date.

The body weight of each animal was monitored before surgery and for a period of one week post-surgery. The number of animals assigned to Sham and SDA surgery was $N = 8$ and $N = 10$, respectively.

2.3. Functional verification of SDA completeness

SDA completeness was verified with an established functional test using exogenous, intraperitoneally (i.p.) administered cholecystokinin (CCK). Intraperitoneally administered CCK depends on intact abdominal vagal afferent fibers (Smith and Gibbs 1985) to induce satiation. The failure of i.p. CCK induced satiation therefore ascertains the completeness of the abdominal deafferentation (SDA). The CCK test was conducted only after the animals had completed the cognitive tests (see below). Exogenous CCK administration therefore did not interfere with the cognitive testing of the animals. The functional verification test of complete abdominal deafferentation was performed as described previously (Klarer et al. 2014). In short, the animals were food deprived overnight (13 hrs) and injected i.p. with 4 µg/kg CCK-8 (Bachem, Bubendorf, Switzerland) or vehicle (phosphate-buffered saline [PBS]) using a within-subject crossover design. The injection of CCK and vehicle, respectively, took place immediately before dark onset of the reversed 12:12 hrs dark–light cycle, after which all rats had free access to their food cups. Individual food intake was measured after 30 min.

CCK-8 treatment in Sham rats typically leads to a robust reduction in food intake within the first 30 min after food presentation (Arnold et al. 2006; Klarer et al. 2014; Labouesse et al. 2012; Porter et al. 1998). Therefore, the inclusion criterion for SDA rats was set at 30% ± SEM of CCK-induced reduction in food intake during the first 30 min. Only data from animals that passed this functional verification were included in the final analysis and presentation of data. Using this criterion, only one out of 10 SDA rats had to

be excluded from the final analysis (see Figure 1***C***), so that the final number of animals was *N* = 8 and *N* = 9 for Sham and SDA, respectively.

2.4. Experimental test order and conditions

Cognitive testing commenced two weeks post-surgery and was conducted during the animals' active phase, that is, during the dark phase of the reversed light-dark cycle between 10h00 and 18h00. The time of daily testing was counterbalanced across the two experimental groups. Throughout all testing, the animals were housed in groups of 2-4 rats per cage in order to avoid confounds arising from isolation stress (Zhang et al. 2012). All rats were tested repeatedly in a series of behavioral tests with an inter-test recovery phase of at minimum 1 to 3 days. The order of cognitive tests was designed in such a way that the test series commenced with the least stressful paradigm and ended with more stressful tests. Hence, the following test order was used: 1. working memory in a Y-maze spontaneous alternation test; 2. novel object recognition test with three different delays; 3. positively reinforced left-right discrimination and reversal learning test; and 4. incentive runway test (see below).

Upon completion of the behavioral analyses, animals were single caged for the functional test ascertaining the lack of CCK satiation. Immediately after completion of the CCK test, the animals were recaged to the original groups of 2-4 animals per cage.

2.5. Working memory in a Y-maze spontaneous alternation test

Working memory was assessed using a spontaneous alternation test in the Y-maze. This test is based on the innate tendency of rodents to explore novel environments (Deacon and Rawlins 2006), that is, their preference to investigate a new arm of the maze rather than returning to one that was previously visited. The Y-maze apparatus consisted of three identical black Plexiglas arms (46 cm × 16 cm; length x width) surrounded by 25-cm high black Plexiglas walls. The arms were aligned in a 120° angle from a center triangle platform. The entire maze was covered with a transparent Plexiglas lid. The maze was

elevated 81 cm above floor level and placed in a dimly lit testing room (~5 lux in the center zone of the maze). A digital camera was mounted above the Y-maze. Images were captured at a rate of 5 Hz and transmitted to a computer running the Ethovision (Noldus Technology, Wageningen, The Netherlands) tracking system, which calculated the total distance moved, and the number of entries into the three arms and the center zone of the Y-maze.

The spontaneous alternation task in the Y-maze was slightly modified from previously established procedures (Krstic et al. 2012). The animals were gently introduced into the start arm and allowed to freely move in the entire Y-maze. The number and sequence of arm entries (defined as the entry of all four paws into one arm) were recorded during a period of 5 min. Alternation was defined as entry into all three arms in any non-repeating order (i.e., ABC, BAC, CBA). The percentage alternation was calculated as the total number of alternations divided by the maximal possible alternations ('total number of arm entries'-2). In addition, the total distance moved and the total number of arm entries were analyzed to assess general locomotor and exploratory activity.

2.6. Novel object recognition test with three different delays

The novel object recognition test (NORT) is based on the innate tendency of rodents to spend more time exploring a novel object than a familiar one, whereby increased exploration of the former reflects recognition memory when given the choice to explore both objects simultaneously (Antunes and Biala 2012). It was conducted in a square arena (80 cm x 80 cm) made of gray Plexiglas and surrounded by 50 cm high walls. The arena was positioned in a testing room with dimly diffused lighting (~15 lux as measured in the center of the arena). The test was conducted with three distinct delays: 1) a minimal delay of 5 min, 2) an intermediate delay of 1 hr, and 3) a long delay of 24 hrs. Different delay conditions were assessed on distinct testing days, separated by 6-8 days. Distinct sets of objects were used for each delay condition. Objects pairs differed in shape,

appearance, and material. The different objects and assignment of familiar and novel object were counterbalanced between groups. A digital camera was mounted directly above the apparatus, and each test trial was recorded for subsequent video analyses by an experimenter, who was blind to the experimental conditions.

All rats were first familiarized to the testing arena two days prior to the actual test. The NORT consisted of two phases, which are referred to as the initial sample and subsequent test phases. In the sample phase, rats were gently placed in the arena where they were presented two identical diagonally placed objects, which they could freely explore for a total of 5 min. Upon completion of the sample phase, the animals were removed and put back into their holding cage. The objects and arena were cleaned between trials to avoid olfactory cues. In the test phase, one of the two familiarized objects was replaced by a novel object, and the rats were gently introduced to test apparatus again after a defined delay between the two phases, allowed to freely explore the objects for 5 min. Physical interaction with objects was defined as sniffing or touching the objects with nose and/or forepaws (Mello-Carpes and Izquierdo 2013). Recognition memory was indexed by the relative time spent exploring the novel versus the familiar object.

2.7. Positively reinforced left-right discrimination and reversal learning

Cognitive flexibility was tested in a positively reinforced left-right (L vs. R) discrimination reversal-learning task in an opaque Y-maze using palatable food (chocolate pellets) as reward. In this paradigm, the animals first learn to respond differentially (typically approaching or avoiding) to two stimuli of opposing valence (i.e., L+ vs. R−), and are then confronted with the same two stimuli but with the reversed valence (i.e., L− vs. R+). The ability to recognize an unexpected consequence from a previously established associative learning rule and then to switch the response contingency accordingly is crucial to reversal learning (Nilsson et al. 2015). The apparatus consisted of an opaque Y-maze as described in section *2.5*. It was positioned in a dimly lit room (~5 lux in the center zone of

the maze). Animals were habituated over three days to a 6 hrs food deprivation and the subsequent presentation of chocolate pellets as reward. Rats were familiarized to the food reward in order to avoid food neophagia (familiar food in novel environment) and neophobia (novel food in familiar environment).

In acquisition training, the animals were required to learn to discriminate the left and right goal arm, with only one of them leading to a food reward placed at the far end of the arm. For one-half of the animals in each group, the left arm was correct, and for the other half, the right arm was correct during acquisition training. The food reward location remained unchanged until the end of acquisition. There were 6 trials per daily session, conducted at an ITI of 10 min. To begin a trial, the animals were introduced into the start arm of the Y-maze and allowed up to 1 min to choose between the two arms. Once its entire body had entered into an arm, a blocking door was used to prevent the animal from retracting. If the rat chose the correct arm, it was rewarded with chocolate pellets, which were presented only after the rat had entered in order to prevent olfactory cues. When the incorrect arm was chosen, the animal was confined to the arm for 20 sec and then removed to the holding cage. Acquisition training continued until an animal had reached criterion performance of 10 correct trials over 2 consecutive days (i.e., 10 correct out of 12 trials). Upon reaching the acquisition criterion, the location of the reward was moved to the previously incorrect arm to assess reversal learning. Reversal training continued until an animal had reached criterion performance once again. The percentage of correct arm choices and the errors to criterion were recorded manually and calculated for each animal during acquisition and reversal training.

2.8. Incentive runway test

The incentive runway test was conducted to assess positively reinforced goal-directed behavior (Ettenberg 2009; Pecina et al. 2003). The apparatus consisted of a square start box (20 cm) and an identical goal box connected with a 180 cm long running alley enclosed by grey Plexiglas walls (50 cm height). The runway was placed in a testing room

with dimmed illumination (~15 lux in running alley). A digital camera was mounted directly above the apparatus and each trial was recorded.

The incentive runway was slightly modified from previously established procedures (Guzman et al. 2009; Pecina et al. 2003; Shin et al. 2011). Rats were first habituated to the food reward (Frosties® cereals, Kellogg's) in their home cages, followed by habituation to the running alley during two daily 10-min sessions. During two additional sessions, overnight food-deprived (16 hrs) rats were enticed to eat a small food reward (2 g Frosties) in the goal box. Runway behavior was then assessed in a minimum food-deprived state (4 hrs) in daily sessions on 5 consecutive days. After placing the animals in the start box, a door was opened and the time to reach the goal box and to start consuming the reward (completion time) was measured and expressed as completion speed (cm/s).

2.9. Statistical analyses

Body weights were analyzed using a 2 × 7 (surgery × days) repeated-measures (RM) parametric analysis of variance (ANOVA). The CCK-induced reduction in food intake was expressed as percentage basal food intake and analyzed using Student's *t* tests (two tailed). All data obtained in the spontaneous alternation test were also analyzed using Student's *t* tests (two tailed). All dependent measures in the NORT were analyzed using 2 × 3 (surgery × delay condition) ANOVAs. Percent correct trials during the acquisition of the positively reinforced left-right discrimination test were analyzed using a 2 × 15 (surgery × days) RM-ANOVA, and the data obtained in the reversal phase were analyzed using a 2 × 13 (surgery × days) RM-ANOVA. The number of total errors during acquisition and reversal were each analyzed using Student's *t* tests (two tailed). Completion speed was analyzed using a 2 x 5 (surgery x days) RM-ANOVA. All ANOVAs were followed by Fisher's least significant difference *post hoc* comparisons or restricted ANOVAs whenever appropriate. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the statistical software IBM SPSS Statistics (version 22).

3. Results

3.1. Body weights and functional verification of SDA

Figure 1*B* shows body weights before SDA or Sham surgeries and during the postsurgical recovery phase. Following an initial drop in body weight induced by the surgical procedures, all animals regained their pre-surgery body weight within 4 days after surgery (**Figure 1*B***). There were no group differences in body weight between rats undergoing SDA or Sham surgery at any pre- or post-surgical time points (**Figure 1*B***).

SDA completeness was functionally verified. As expected (Arnold et al. 2006; Azari et al. 2014; Klarer et al. 2014; Labouesse et al. 2012; Porter et al. 1998), Sham rats showed a 50-60% ($p < 0.001$) reduction in food intake after CCK application relative to their baseline food intake (**Figure 1*C***). Nine out of 10 SDA rats displayed no such reduction in food intake in response to CCK administration relative to their baseline food intake, confirming the completeness of SDA in the animals. One SDA rat, however, showed a 45.8% reduction in food intake relative to its baseline food intake in response to CCK administration (see **Figure 1*C***) and was therefore excluded from the final analyses due to the presumed lack of SDA completeness. Hence, the total number of animals included in all cognitive test was $N = 9$ for SDA rats, and $N = 8$ for Sham rats.

3.2. Effects of SDA on working memory

We first assessed the effects of SDA on measures of working memory. Working memory is a special short-term memory buffer with limited temporal capacity and is used to hold relevant information active in order to guide on-going behavior (Baddeley 2000). The spontaneous alternation task is based on the innate tendency of rodents to explore novel environments, that is, their preference to investigate a new arm of the maze rather than returning to one that was previously visited (Deacon and Rawlins 2006).

As shown in **Figure 2*A***, the percent alternation score was ~ 70% in both Sham and SDA rats. There were also no group differences in terms of the total number of arm entries (**Figure 2*B***) or total distance moved (**Figure 2*C***) during the test. Hence, SDA did not affect measures of working memory or general exploratory activity in the spontaneous alternation test.

3.3. Effects of SDA on recognition memory

To examine whether abdominal vagal deafferentation may affect recognition memory, we compared SDA and Sham rats in a two-object novel object recognition test (NORT). This test is based on the innate tendency of rodents to spend more time exploring a novel object than a familiar one, as indexed by the relative time spent exploring the novel versus the familiar object. The NORT consisted of two phases, which are referred to as the initial sample and subsequent test phases. In the sample phase, rats were presented two identical objects in an open field arena, whereas in the test phase, one of the two familiarized objects was replaced by a novel object.

As shown in **Figure 3A**, there were no group differences in terms of total object exploration during the initial sample phases, indicating that SDA did not affect object exploration per se. Likewise, general locomotor activity (as indexed by the total distance moved) during the initial sample phases did not differ between SDA and Sham rats (**Figure 3B**). When given the choice to simultaneously explore the novel and familiar objects in the test phase, both Sham and SDA rats displayed a clear preference towards the novel object (**Figure 3C**). Hence, there were no group differences in terms of the relative time spent with the novel object at any of the three delay conditions, indicating that SDA did not affect recognition memory. The relative time spent with the novel object generally decreased with increasing delays between sample and test phases (main effect of delay: $F_{(2,30)} = 3.64$, $p < 0.05$), leading to a significant ($p < 0.05$) difference between the 5-min and 24-hrs delay condition (**Figure 3C**). At the same time, the total distance moved

during the test phase increased as a function of delays (main effect of delay: $F_{(2,30)}$ = 15.43, p < 0.001), so that the total distance moved was longer (p < 0.05) in the 24-hrs delay condition compared to the 5-min (p < 0.001) or 1-hr (p < 0.01) delay condition. These delay-dependent effects emerged independently of the between-subjects factor of lesion.

3.4. Effects of SDA on discrimination and reversal learning

In a next step, we examined whether abdominal vagal deafferentation may affect measures of cognitive flexibility. To this end, we used a positively reinforced left-right (L vs. R) discrimination reversal-learning task using palatable food (chocolate pellets) as reward. In this paradigm, the animals first learn to respond differentially (typically approaching or avoiding) to two stimuli of opposing valence (i.e., L+ vs. R−), and are then confronted with the same two stimuli but with the reversed valence (i.e., L− vs. R+).

For the initial acquisition training, the animals were required to learn to discriminate the left and right goal arms, with only one of them being rewarded and continued until the animals had reached criterion performance. Sham and SDA rats performed similarly during the acquisition phase of the test, and both groups reached criterion within 15 days of acquisition training (**Figure 4**). In both groups, the percent correct trials increased as a function of training days, leading to a significant main effect of days ($F_{(14,210)}$ = 11.85, p < 0.001). Upon reversal of the response contingencies, however, SDA rats acquired criterion faster than Sham controls (**Figure 4**). Indeed, SDA rats displayed an increase in the percentage of correct trials (main effect of lesion: $F_{(1,15)}$ = 7.76, p < 0.05) and a decrease in the total number of errors (p < 0.01) to reach criterion during the reversal phase of the test. Together, these findings show that SDA selectively enhances positively reinforced reversal learning without affecting left-right discrimination learning per se.

3.5. Effects of SDA on positively reinforced goal-directed behavior

We then investigated whether the effects of SDA on positively reinforced reversal learning (see **Figure 4**) might be confounded by changes in the motivation to obtain natural rewards. Therefore, we compared SDA and Sham rats in the incentive runway test, which assesses positively reinforced goal-directed behavior. In order to exclude potential confounds arising from food neophobia (Klarer et al. 2014), all rats were familiarized with the food reward (Frosties® cereals, Kellogg's) in their home cages before they were then habituated to the goal box containing 1g of the same reward. Both experimental groups showed similar latencies to start eating (Sham: 23.4 ± 2.8, SDA: 23.0 ± 3.7; $p = 0.94$) and finishing (Sham: 87.4 ± 5.2, SDA: 96.1 ± 7.0; $p = 0.34$) the reward during the goal-box habituation phase.

After habituation, runway behavior was assessed in daily sessions on 5 consecutive days. Consistent with previous rodent studies (Guzman et al. 2009; Pecina et al. 2003; Shin et al. 2011), the completion speed increased as a function of testing days, leading to a significant main effect of days ($F_{(4,60)} = 21.75$, $p < 0.001$). The completion speed equally rose in SDA and Sham rats as a function of training days (**Figure 5**), indicating that positively reinforced goal-directed behavior in the incentive runway test was not affected by SDA.

4. Discussion

Using the most complete and selective subdiaphragmatic vagal deafferentation model existing to date, our study sought to examine the functional contribution of abdominal vagal afferent signaling to various cognitive functions. We found that SDA did not affect working memory in a nonspatial alternation task, nor did it influence short-, intermediate-, and long-term object recognition memory. On the other hand, SDA facilitated reversal learning in a positively reinforced left-right discrimination task without affecting

discrimination learning per se. The selective effect of SDA on reversal learning is unlikely to be mediated by altered motivation towards positive reinforcers or confounded by altered neophobic behavior or physiological needs for food. In support of this notion, SDA and Sham rats did not differ during the initial acquisition phase of the reversal learning test. Moreover, SDA and Sham animals showed a completion speed in the incentive runway test, which assesses positively reinforced goal-directed behavior (Guzman et al. 2009; Pecina et al. 2003; Shin et al. 2011). Importantly, SDA and Sham animals also displayed similar latencies to start eating and finishing the food reward in the incentive runway test, suggesting fully extinguished neophobic behavior and similar physiological needs for food in all rats. Hence, the SDA-induced facilitation of reversal learning likely reflects a genuine enhancement of cognitive flexibility, whereby SDA animals display a more rapid behavioral adaptation to changing contingencies. Taken together, our data indicate that the relative contribution of vagal afferent signaling arising from abdominal viscera to cognitive functions is restricted to, or most evident in, conditions that require subjects to adapt their cognitive strategies to face new and unexpected changes in the environment.

Several reports have identified enhancing effects of VNS on cognition including recognition memory (Clark et al. 1999) and working memory (Beste et al. 2016; Hansen et al. 2003), suggesting that the vagus nerve may play an endogenous role as a mediator of cognition. Whilst our findings do not support this hypothesis, they corroborate previous human studies showing that activation of vagal afferents by VNS impairs cognitive flexibility without inducing general deficits in various forms of learning and memory (Ghacibeh et al. 2006a; 2006b). Hence, whereas a stimulation of vagal afferent signaling impairs cognitive flexibility, its disruption by SDA leads to diametrically opposite effects. These findings collectively suggest that visceral signals can influence cognitive flexibility in a bidirectional manner, depending on whether vagal afferent pathways are stimulated or inhibited.

It has long been recognized that reversal learning is critically dependent on prefrontal cortical functions, especially within the orbifrontal cortex (OFC) and medial

prefrontal cortex (mPFC) (Clark et al. 2004; Hamilton and Brigman 2015; Ragozzino and Rozman 2007). Whereas the OFC is crucial for simple forms of reversal learning, including visual discrimination learning tasks involving stimulus-outcome contingencies, the mPFC seems to be more directly involved in reversal learning tasks that require movement through space (Hamilton and Brigman 2015; Ragozzino and Rozman 2007) or for solving serial discrimination problems (Johnson and Wilbrecht 2011; Kennerley et al. 2006; Ragozzino and Rozman 2007). In our testing conditions, the animals were trained to acquire and reverse positively reinforced responses in a Y-maze apparatus, which required them to move through a defined spatial environment. Given these settings, we believe that the SDA-induced changes in reversal learning may, at least in part, involve functional changes in the mPFC region.

In support of this hypothesis, we recently showed in the same model that SDA causes neurochemical imbalances in the mPFC (Klarer et al. 2014). More specifically, we found decreased NA and increased GABA levels in the mPFC of SDA rats compared to Sham controls (Klarer et al. 2014). These neurochemical imbalances likely represent secondary responses to primary changes in up-stream neuronal signaling pathways that are directly targeted by vagal afferents. The primary target region of vagal afferents is the NTS, from where visceral signals are further conveyed to brainstem nuclei, limbic structures and cortical regions, including the mPFC (Barnes et al. 2003; Berthoud and Neuhuber 2000; Khalsa et al. 2009). For example, the NTS directly innervates the locus coeruleus (LC), a brain area harboring the majority of noradrenergic neurons in the CNS (Mello-Carpes and Izquierdo 2013). LC noradrenergic neurons, in turn, project to various forebrain structures, including the mPFC (Naritoku et al. 1995). On speculative grounds, the SDA-induced decrease in prefrontal NA may thus be caused by lower stimulation of NTS neurons, which would result in decreased activation of LC neurons and reduced noradrenergic outputs in forebrain structures such as the mPFC. Consistent with this notion, chronic VNS has been shown to enhance the firing rate of NA neurons in the LC (Dorr and Debonnel 2006; Groves et al. 2005) and to increase NA concentrations in

subcortical and cortical areas, including the mPFC (Follesa et al. 2007; Groves et al. 2005; Manta et al. 2013; Roosevelt et al. 2006). On the other hand, the neuroanatomical routes that are responsible for modulating GABA levels in the mPFC of SDA rats remain largely elusive and warrant investigations in future studies.

The SDA-induced changes in prefrontal NA may be of particular relevance for explaining the lesion's effect on reversal learning. Prefrontal NA has long been implicated in the regulation of attentive processes, arousal, and vigilance, all of which are crucial for cognitive and behavioral flexibility (Beversdorf et al. 2002; Bouret and Sara 2005; Dalley et al. 2004; Kehagia et al. 2010; Sara and Bouret 2012). It has further been suggested that NA influences cognitive flexibility by altering prefrontal signal-to-noise ratios and by its ability to modify the brain's capacity to attend to sensory inputs (Ramos and Arnsten 2007). One of the main neuronal effects of NA is to suppress the synaptic potentials elicited on intrinsic fiber layers (Aston-Jones et al. 1991; Hasselmo et al. 1997). Thus, reduced NA signaling in the mPFC readily acts to increase transmission between pyramidal cells, thereby enhancing intrinsic prefrontal cortical activity. In addition to these effects, NA can modify the influence of afferent inputs onto cortical neurons (Aston-Jones et al. 1991; Hasselmo 1995). Collectively, one of the consequences of decreased phasic NA release is that it enhances the cross-communication between cortical neurons, which in turn increase in the relative size of the cortical networks used to process information (Aston-Jones et al. 1991; Hasselmo 1995; Kischka et al. 1996). The engagement of large-scale cortical networks would then permit a more rapid behavioral adaptation to changing environmental cues or contingencies (Bouret and Sara 2005; Dayan and Yu 2006; Kischka et al. 1996; Yu and Dayan 2005), such as seen in SDA rats displaying enhanced discrimination reversal learning. Similar NA-related processes have also been proposed to underlie the disruption of cognitive flexibility by VNS (Ghacibeh et al. 2006a). In the latter case, VNS-induced increases in NA activity would increase the signal-to-noise ratio and improve the brain's ability to attend to sensory inputs. At the same time, however, it would

decrease the ability to recruit large-scale networks necessary for rapid behavioral adaptations to changing environments (Ghacibeh et al. 2006a).

Despite the consistencies between some of the effects induced by SDA and VNS, it should be noted that abdominal vagal deafferentation cannot be assumed to produce effects that are generally opposite to those of VNS. Therefore, gain of cognitive functions induced by VNS would not necessarily mean that SDA would generally cause impairments in similar cognitive domains. In fact, the vagus nerve may simply have a modulatory role in cognition, so that its stimulation (by VNS) may act supportive under certain conditions only. These considerations may help to explain why we did not see clear effects of SDA on working memory or object recognition memory, unlike those studies that used VNS (Beste et al. 2016; Clark et al. 1999; Hansen et al. 2003). The SDA-induced effects on reversal learning, however, add further weight to theories emphasizing an important role of afferent vagal signaling originating from the abdominal viscera in the regulation of certain behavioral and cognitive functions. Using the same model of SDA in rats, we previously showed that abdominal vagal afferents modulate innate anxiety and learned fear, suggesting a functional role of afferent visceral signaling in emotional behavior (Klarer et al. 2014).

Notably, SDA-induced effects on positively reinforced reversal learning seem to be incompatible with the attenuation of conditioned fear extinction as previously demonstrated in the same model of abdominal vagal deafferentation (Klarer et al. 2014). Positively-reinforced reversal learning and extinction of cued fear conditioning, however, critically differ in terms of the underlying neuropsychological mechanisms and conceptual processes. Firstly, there are clear differences between these two tests with regards to the intrinsic valence of the test stimuli. Whereas cued fear conditioning is mediated by aversive stimuli (negative valence), the current test of discrimination and reversal learning in the Y-maze is largely driven by rewarding stimuli (positive valence). In keeping with this valance dichotomy, SDA rats seem to display a more rapid behavioral adaptation to changes in contexts or stimuli with positive valence, whereas they may adapt slower to

changes in contexts or stimuli with negative valance. Secondly, positively-reinforced reversal learning and cued fear conditioning also differ conceptually, which in turn may shape the direction of the effects induced by SDA. Auditory-cued fear conditioning requires that a consequential stimulus (i.e., the unconditioned stimulus; e.g., electric foot shock) is signaled and therefore predicted by an initially neutral stimulus (i.e., conditioned stimulus; e.g., tone). In contrast, the presence of reward in the positively reinforced left-right discrimination test is not signaled by any discreet stimulus. Hence, even though both tests seem to involve distinct phases of acquisition and extinction, they critically differ with respect to the extent to which a consequential stimulus (food reward or aversive foot shock) can be predicted by another (initially neutral) stimulus.

An emerging question that awaits exploration is whether the behavioral and cognitive consequences of SDA may reflect a failure of the organism to convey gut-derived signals form the viscera to the CNS (Cryan and Dinan 2012). Indeed, accumulating evidence indicates that visceral signals, such as those stemming from gut microbiota, are crucial for normal brain development and functions (Bravo et al. 2011; Diaz Heijtz et al. 2011; Fröhlich et al. 2016; Hoban et al. 2016a), including those involving prefrontal structures (Hoban et al. 2016b). Intriguingly, the selective effects of SDA on cognitive flexibility are reminiscent of and consistent with the recent findings obtained in a mouse model of diet-induced gut dysbiosis, which identified a clear association between poorer cognitive flexibility and altered gut microbiota composition (Magnusson et al. 2015).

As discussed in detail elsewhere (Arnold et al. 2006; Norgren and Smith 1994), SDA leads to complete disconnection of all abdominal vagal afferents whilst sparing half of the vagal efferents. Hence, half of the vagal efferents are disconnected by the SDA procedure, which in turn may complicate the interpretations of the relative contribution of vagal afferents versus efferents to the observed effects on reversal learning. Unfortunately, complete and uniquely vagal deafferentation at the cervical level through bilateral rhizotomy is at present not practically feasible as it causes severe breathing

complications leading to lethality within 48 h post-surgery (M. Arnold; unpublished observations). Hence, the SDA procedure used here is still the most complete and selective vagal deafferentation method existing to date. Another limitation of our study is that the cognitive effects of SDA were assessed in male rats only. Hence, possible sex-dependent effects of SDA on cognitive functions could not be examined herein. Finally, the current interpretation suggesting that SDA enhances cognitive flexibility should be met with some caution as it is based on one particular test only. It would therefore be desirable that future studies further compare SDA and Sham rats in other paradigms assessing cognitive flexibility and related executive functions.

In conclusion, the present study shows for the first time that cognitive flexibility, at least in the domains of positively reinforced learning, is subjected to visceral modulation through abdominal vagal afferents. Future investigations of the possible links between gut-derived signals, their vagal afferent communication to the brain, and/or neurochemical imbalances in prefrontal areas will readily help to gain more insights into the intricate mechanisms whereby visceral signals can influence the adaption of cognitive strategies in changing environments.

5. Acknowledgements

This work was supported by funding granted by ETH Zurich, Switzerland (ETH Research Grant ETH-25_13-2) awarded to U.M. and W.L. The authors declare no competing financial interests.

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Figure Legends

Figure 1. *(A)* Schematic illustration of afferent and efferent vagal fibers targeted by the subdiaphragmatic vagal deafferentation (SDA) procedure. Afferent and efferent fibers are represented in red and blue colors, respectively. In the SDA procedure, the dorsal (right) subdiaphragmatic trunk of the vagus nerve is fully transected (indicated by the first scissor symbol), leading to a disconnection of both afferent and efferent fibers in the dorsal trunk of the vagus nerve. The ventral (left) subdiaphragmatic trunk of the vagus nerve is left intact at this level. In addition, a left-sided intracranial vagal rhizotomy is performed (indicated by the second scissor symbol) to selectively disconnect the remaining vagal afferents. This SDA procedure induces a complete (100%) disconnection of vagal afferents in the abdomen while leaving 50% of the abdominal vagal efferents functionally intact. Vagally innervated organs: H- heart, L- lung, Liv- liver, Sto- stomach, P- pancreas, DJI- duodenum, jejunum, ileum, C- colon, Sp- spleen, Ki- kidney. *(B)* Body weights before surgery (day 0; indicated by the dashed lines) and during the first recovery week (days 1-7). Note that both Sham and SDA rats similarly regained their pre-surgery body weight similarly within 4 days after surgery. No significant group differences were detected between SDA and Sham rats. *(C)* Verification of SDA completeness in a cholecystokinin (CCK) satiation test. The bar plot shows the percentage (%) reduction in CCK-induced food intake (% baseline food intake) during a 30 min test session. Note that SDA rats displayed only minimal or no reduction in food intake in response to CCK administration relative to their baseline food intake, confirming the completeness of SDA. In contrast, Sham control rats displayed a 50-60% reduction in food intake in response to CCK relative to their baseline food intake. Against these backgrounds, one SDA animal (with 45.8% reduction in food intake in response to CCK, indicated by the red circle) was excluded from the final analysis and presentation of the data. ***$P < 0.001$. N(Sham) = 8, N(SDA) = 9. All values are means ± SEM.

Figure 2. No effects of SDA on working memory in a Y-maze spontaneous alternation task. *(A)* Percent alternation (%) in SDA and Sham control rats. *(B)* Total number of arm entries made by SDA and Sham control rats. *(C)* Total distance moved (cm) in SDA and Sham control rats. *N*(Sham) = 8, *N*(SDA) = 9. All values are means ± SEM.

Figure 3. No effects of SDA on novel object recognition memory. *(A)* Total object exploration times (s) for SDA and Sham control rats during the sample phases preceding the test phases under minimal (5 min), intermediate (1 h), and long (24 h) delay conditions. *(B)* Total distances moved (cm) for SDA and Sham control rats during the sample phases preceding the test phases under minimal (5 min), intermediate (1 h), and long (24 h) delay conditions. *(C)* Relative times spent with the novel object during the test phases under minimal (5 min), intermediate (1 h), and long (24 h) delay conditions. Note that all animals clearly performed above chance level (as indicated by the dashed line) in each delay condition. *$P < 0.05$, reflecting the general reduction in relative times spent with the novel object under the long (24 h) versus minimal (5 min) delay condition. *(D)* Total distances moved (cm) during the test phases under minimal (5 min), intermediate (1 h), and long (24 h) delay conditions. **$P < 0.01$ and ***$P < 0.001$, reflecting the general increase in total distance moved under the long (24 h) versus minimal (5 min) or intermediate (1 h) delay condition. *N*(Sham) = 8, *N*(SDA) = 9. All values are means ± SEM.

Figure 4. Effects of SDA on positively reinforced left-right discrimination and reversal learning. *(A)* Percent correct trials as a function of testing days during the initial acquisition and subsequent reversal phase. The dashed line represents chance level. *$P < 0.05$, reflecting the general increase in percent correct trials in SDA relative to Sham control rats during the reversal phase. *(B)* Total number of errors during the initial acquisition (Acq) and subsequent reversal (Rev) phase. **$P < 0.01$, reflecting the decrease in number of

errors in SDA relative to Sham control rats during the reversal phase. *N*(Sham) = 8, *N*(SDA) = 9. All values are means ± SEM.

Figure 5. No effects of SDA on positively reinforced goal-directed behavior. Completion speed (cm/s) in the incentive runway test as a function of successive test days in SDA and Sham control rats. Completion speed was calculated based on time leaving the start box, reaching the goal box, and starting eating food reward. Completion speed similarly increased in SDA and Sham rats. *N*(Sham) = 8, *N*(SDA) = 9. All values are ± SEM.