Norepinephrine Transporter Blocker Atomoxetine Increases Salivary Alpha Amylase

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Contributors

CMW, SN, and JAB designed the study. CMW and RLvdB collected the data. CMW, RLvdB, and JAB processed the data. CMW analyzed the data. CMW and RLvdB made the figures. All authors contributed to writing the paper, and approved the final version. This work was supported by a Consolidator Grant of the European Research Council.

Conflicts of interest: none

Highlights

- Healthy adults received 1 dose of atomoxetine, which increases norepinephrine levels
- Atomoxetine increased salivary alpha amylase
- Atomoxetine increased salivary cortisol, replicating previous work
- Robust correlation between treatment effects on salivary alpha amylase and cortisol

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Abstract

It has been suggested that central norepinephrine (NE) activity may be inferred from increases in salivary alpha-amylase (SAA), but data in favor of this proposition is limited. We administered 40 mg of atomoxetine, a selective NE transporter blocker that increases central NE levels, to 24 healthy adult participants in a double-blind, placebo-controlled cross-over design. Atomoxetine administration significantly increased SAA secretion and concentrations at 75 to 180 minutes after treatment (more than doubling baseline levels). Consistent with evidence that elevation in central NE is a co-determinant of hypothalamic-pituitary-adrenal axis activity, salivary cortisol also approximately doubled at the same time points. Moreover, changes in salivary cortisol positively correlated with SAA ($.44 < rho < .56$), bolstering the position that the origin of the changes in SAA reflect central NE. This work points toward the potential value of SAA as an inexpensive and non-invasive procedure to obtain information about activation of the central NE system.

Keywords:

Atomoxetine, alpha amylase, noradrenaline, sympathetic, yohimbine, locus-coeruleus, noradrenergic

1. Introduction

Norepinephrine (NE) is one of the most widely distributed neuromodulators in the mammalian brain, playing a central role in many cognitive processes, and disturbances of the NE system have been implicated in a variety of neuro-psychiatric conditions (Berridge & Waterhouse, 2003). However, a recognized limitation to further development of functional theory and translation into clinical practice is the absence of practical markers of central NE activity for use in human subjects.

Ehlert and colleagues (2006) have proposed salivary alpha amylase (SAA) as a candidate marker of central NE activity. SAA is a digestive enzyme that is released by the salivary glands in response to stimulation by the local sympathetic nerves. This activity, in turn, has the central locus coeruleus-norepinephrine system as a main determinant (Dunn, Swiergiel, & Palamarchouk, 2004). In a placebo-controlled design, Ehlert and colleagues (2006) observed copious release of SAA in 13 participants administered with yohimbine, an α2-adrenergic receptor antagonist that increases peripheral and central NE. Based on the observation that stress-induced increases in SAA did not correlate with peripheral release of plasma NE, Ehlert and colleagues reasoned that SAA more likely reflects the actions of central NE activity. While these are intriguing and potentially important findings, currently they reflect the only evidence to support the notion of SAA as a marker of central NE.

Complicating the interpretation of the above data is that yohimbine also has strong parasympathetic effects on the salivary glands, through blocking of inhibitory α2 receptors on pre-and post-ganglionic salivatory parasympathetic neurons (Samuels & Szabadi, 2008). This effect is evident in a large enhancement of salivary flow rate (e.g. Bagheri et al., 1997), which was also observed by Ehlert and colleagues. These parasympathetic effects present a potential confound for at least two reasons: first,

release of SAA is partially under parasympathetic control, i.e., independent of central regulation of the local sympathetic nerves (Bosch et al., 2011; Proctor & Carpenter, 2007). Second, such parasympathetic effects on SAA release are difficult to detangle from sympathetic effects, as they co-determine SAA release in a non-additive, interactive manner (Proctor & Carpenter, 2007; Bosch et al., 2011; Nagy et al., 2015). Thus, SAA release by yohimbine involves a mix of sympathetic/NE and parasympathetic/cholinergic effects (Berntson, Cacioppo, & Bosch, 2016).

A possible further complication is that the inference that SAA release may involve the actions of central NE was based on negative evidence: i.e., the lack of an association with peripheral catecholamines (Ehlert et al., 2006). However, an absent or weak association between various measures of peripheral sympathetic-adreno-medullary (SAM) activity is a rather common observation, although there exist little doubts about their shared central determinants, and the interpretation of a non-association is thus somewhat uncertain (Bosch et al., 2011; Bosch et al., 2009). Moreover, later reanalysis of the same data showed that peripheral noradrenaline and SAA were, in fact, highly correlated (Ditzen, Ehlert, & Nater, 2014). Thus the proposal by Ehlert and colleagues (2006) would seem to benefit from additional, positive validation.

In light of the points detailed above, the present study sought to examine the relationship between central NE and SAA using atomoxetine, a highly selective NE transporter blocker that raises central NE while exhibiting few side effects compared to other noradrenergic drugs (Bymaster et al., 2002; Chamberlain, et al., 2007).

We administered 40 mg of atomoxetine to 24 healthy adult participants in a randomized, double-blind, placebo-controlled, cross-over design to determine if NE reuptake inhibition would increase SAA release. Relevant to the concerns raised above, we also predicted that increases in SAA release would positively correlate with elevated salivary cortisol. Underlying this prediction is the observation that central (but not peripheral) NE activity is associated with release of cortisol (Bosch et al., 2009); a glucocorticoid

stress hormone that is released upon activation of the hypothalamic-pituitary-adrenal axis, which is anatomically and functionally distinct from the SAM system (Bosch et al., 2009; Hill et al., 2003). This effect of central NE is mediated, in part, by noradrenergic inputs to the hypothalamus (Hill et al., 2003). Consistent with the notion that both SAA and salivary cortisol increases are driven by a common central NE determinant, Chamberlain and colleagues (2007) demonstrated that a 60-mg dose of atomoxetine increases salivary cortisol, and a similar effect has been shown for other NE reuptake inhibitors (Hill et al., 2003).

1. Methods

2.1 Participants

Twenty-four young adult participants (age range 19-26, 5 male) were recruited through campus advertisement as part of a larger neuroimaging study (van den Brink et al., 2016). Participants were screened by a physician for physical health, drug contraindications, and other exclusion criteria (see Supplementary Material). Thirteen of the female participants reported currently using oral contraception. Participants were paid 135 euros for two sessions of 210 minutes each. All participants gave informed consent, and the study was approved by the Leiden University medical ethics committee.

2.2 Procedure

Atomoxetine and placebo were administered in separate sessions in randomized order, spaced one week apart, and scheduled at the same time of day. Atomoxetine was administered orally as a single, encapsulated 40-mg pill, and ingested with water. The placebo consisted of carrier only and was identical in appearance to the active drug. Saliva samples were collected five times per session, beginning immediately before taking the pill (T=0), at T=30, T=75, T=105, and T=180 minutes after pill ingestion. These time points are consonant with the pharmacokinetics of atomoxetine, which reaches a plateau in

blood plasma levels after approximately 90 minutes and remains elevated for up to 9 hours (Sauer, Ring, & Witcher, 2005). In between samples, participants intermittently rested and participated in a resting-state neuroimaging study. We note that undergoing fMRI assessment, which was part of the larger study, may have provoked a stress response in some of our subjects, which might have interacted with the effects of the drug (see Supplementary Material). However, our counterbalanced, randomized, cross-over design would be expected to adequately eliminate any potential of a confounding effect of stress on SAA.

2.3 Saliva sample collection and processing

Whole saliva was collected using the spitting method, whereby participants were asked to let saliva collect passively on the floor of their mouth, and to spit the accumulated saliva into a polypropylene tube every minute over a three-minute period (Beltzer et al., 2010; Ehlert et al., 2006). This method does not elicit confounding flow rate effects (e.g., through mechanical or gustative stimulation) and avoids biasing effects of absorbent materials (Bosch et al., 2011). Fresh samples were stored on ice for a maximum of two hours, homogenized using a vortex mixer, and centrifuged at room temperature for 4 minutes at 4,000 x g to eliminate debris and bacteria. The clear supernatant was aliquoted and frozen at -60 °C until the assay procedure. Samples were assayed for alpha amylase using a quantitative kinetic determination kit (IBL, Hamburg, Germany), essentially as described elsewhere (Nagy et al., 2015). The assay has a sensitivity of 12.5 U/ml, and an intra-assay variability (CV%) of 3.3%. Samples were assayed for cortisol using a competitive enzyme-linked immunosorbent assay, according to the manufacturer's instructions (IBL, Hamburg, Germany), with a sensitivity of 0.045 µg/mL, and intra-assay variability (CV%) of 2.5%. All samples of the same participant were assayed simultaneously.

2.4 Statistical analysis

Saliva data were analysed using a mixed ANOVA with treatment and time as within-subjects factors and treatment order as a between-subjects factor. Including use of oral contraception and sex as categorical variables in the omnibus ANOVA yielded no significant effects of these variables, and did not meaningfully change the reported effects of treatment. Missing data points (e.g., insufficient saliva) were estimated through interpolation by taking the mean of the immediately preceding and subsequent time point (Chamberlain et al., 2007). In the case of a missing baseline (T=0), the baseline value from the other session was used[2]. In all other instances, data points were entered as missing. For SAA, 10/240 points were interpolated and 9/240 were missing, resulting in 21 participants being included in the omnibus ANOVA. For cortisol, 3/240 points were interpolated and 10/240 point were missing, resulting in 22 participants being included in the omnibus ANOVA.

In examining the relationship between drug effects on SAA and salivary cortisol, we computed difference (Δ) scores in two ways. To examine within-session drug effects (drug effect over time), difference scores were calculated by subtracting the baseline (T=0) measure in the atomoxetine condition from the peak value after treatment. To examine between-session drug effects (difference between drug and placebo) difference scores were calculated by subtracting mean concentrations from samples 3-5 (for which significant treatment effects were observed, see below) in the placebo session from those in the atomoxetine session.

2. Results

3.1 Salivary alpha amylase concentration and secretion

[2] In repeated-measures ANOVAs, a single missing data point will result in the elimination of all data from that subject from the analysis. Consequently, ANOVAs without imputation resulted in a large reduction in total N. As a result, some of the interactions reported did not reach significance, although effect sizes remained nearly identical. The analysis of simple main effects, most central to supporting the conclusions of this paper, remained significant without imputation

Atomoxetine increased SAA concentration over time, as compared to the placebo condition, yielding a significant time by treatment interaction, $F(4,72) = 9.03$, $p < .001$ (**Fig. 1a)**. This interaction was largely driven by a steep increase in SAA in the atomoxetine condition. Post-hoc analyses showed that this increase was significantly greater than placebo at T=75 ($t(22) = 3.66$, $p = .001$), and remained larger through to the final sample, T=105 ($t(22) = 3.43$, $p = .002$), and T=180 ($t(20) = 5.97$, $p < .001$).

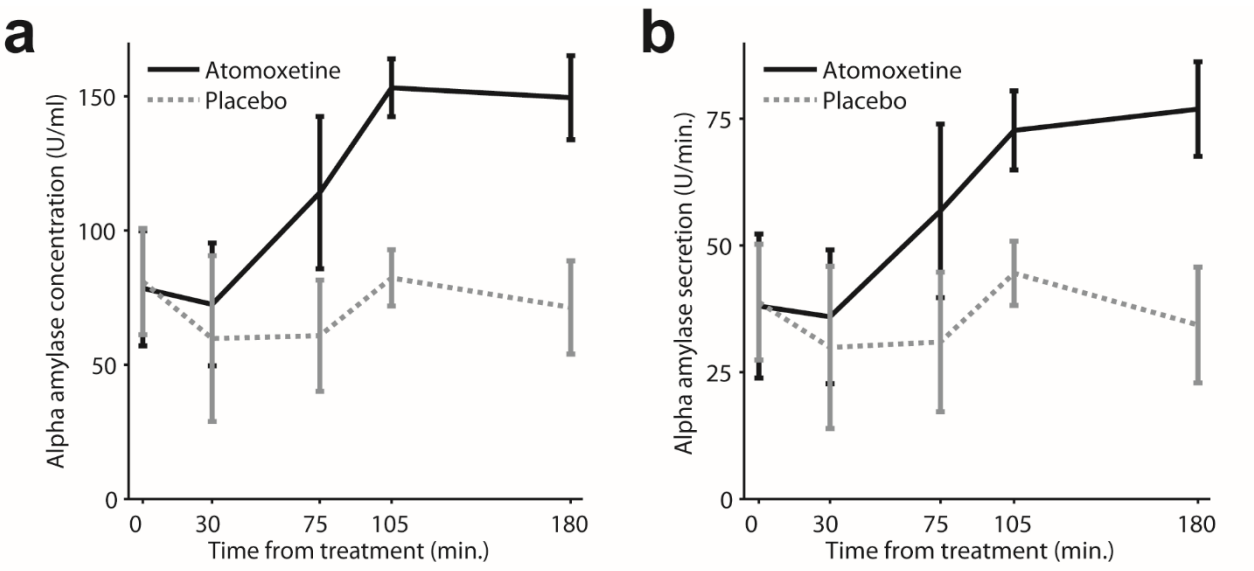

Figure 1. Effect of atomoxetine on salivary alpha amylase (SAA). Time course of treatment and placebo effects on (a) SAA concentrations and (b) SAA secretion (concentration x flow rate). Error bars reflect SEM.

The above findings were replicated after adjusting for salivary flow rate [SAA secretion = SAA concentration X flow rate] (see Supplementary Material). Atomoxetine also increased SAA secretion, resulting in a significant time by treatment interaction, $F(4,72) = 5.49$, $p = .001$ (**Fig. 1b**). As with the concentration data, the effect of atomoxetine on SAA secretion was significantly different from placebo at T=75 ($t(22) = 2.89$, $p = .009$), and held through T=105 ($t(22) = 2.60$, $p = .016$), and T=180 ($t(20) = 4.37$, $p < .001$).

3.2 Cortisol concentrations

Atomoxetine likewise increased salivary cortisol over time relative to the placebo condition, yielding a significant time by condition interaction, $F(4,84) = 8.01$, $p < .001$ (**Fig. 2a)**. Post-hoc analyses again showed that treatment effects on salivary cortisol concentration became apparent at T=75 ($t(21) = 2.55$, $p = .018$), T=105, ($t(21) = 3.57$, $p = .002$), and at T=180 ($t(21) = 4.16$, $p < .001$).

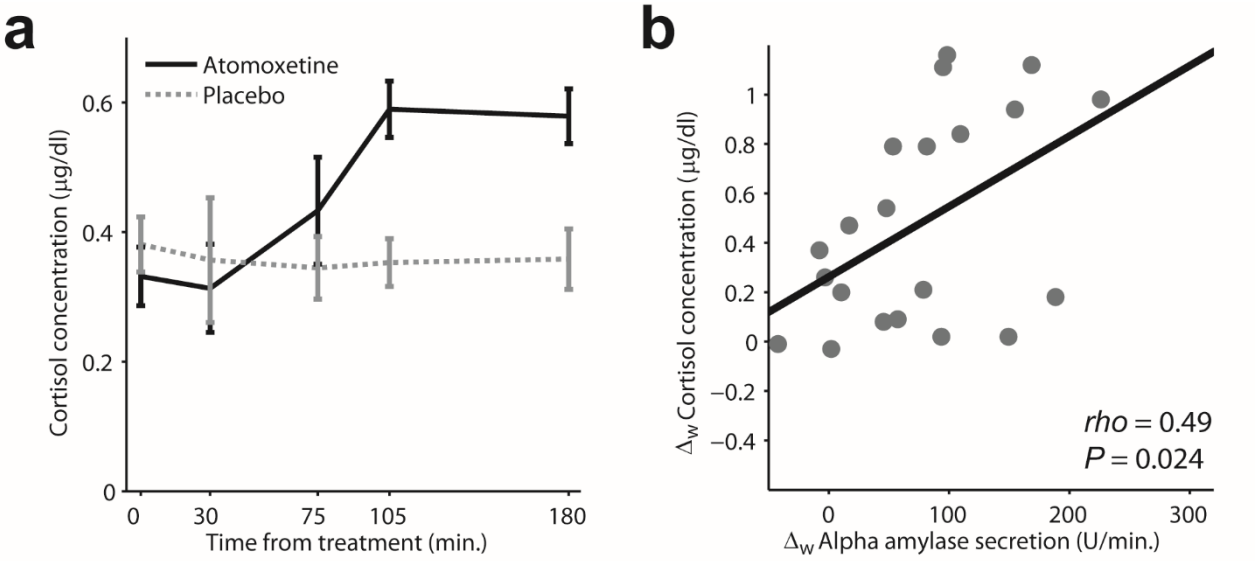

Figure 2. **(a)** ***Effect of atomoxetine on salivary cortisol and (b) relationship between treatment effect on salivary measures of alpha amylase and cortisol. Error bars reflect SEM. Delta values were calculated by subtracting baseline from peak values after treatment.***

3.3 Relationship between salivary alpha amylase and cortisol

We were interested in whether the drug-induced effect on SAA would correlate with the drug-induced effect on cortisol. The Spearman rank correlation (*rho*) between within-session treatment effects on SAA secretion and cortisol concentration was significant, *rho* = .49, $p = .024$ (**Fig. 2b**). Additional figures presented in the online supplement demonstrate a robust relationship between SAA and salivary cortisol: the relationship was also significant when SAA secretion was replaced by SAA concentration, *rho*

= .44, *p* = .047, (**Fig. S1a**), and when correlating between-session treatment effects, *rho* = .52, *p* = .017 (**Fig. S1b**).

3. Discussion

The present study aimed to investigate the effects of the NE transporter blocker atomoxetine on SAA. The results show, for the first time, that atomoxetine enhances SAA concentration and secretion. This result presents a complimentary test of the idea, first advanced by Ehlert and colleagues (2006), that SAA measures can be used to infer central NE activity. The current results also replicate and extend work from Chamberlain and colleagues (2007), showing that atomoxetine increases salivary cortisol at a dose lower than used previously (40 mg vs. 60 mg). In line with predictions, the atomoxetine-induced changes in SAA concentration and secretion correlated with changes in salivary cortisol, for both within-session and between-session treatment effects. This robust association between markers that reflect two physiologically and anatomically distinct stress systems, but share central NE as a common determinant, presents strong convergent evidence that increases in SAA reflect elevations in central noradrenergic activity.

The current study also improves upon prior work by Ehlert and colleagues (2006) by using a drug that has very limited, if any, acute cholinergic/parasympathetic effects. Ehlert and colleagues used yohimbine, a drug with strong cholinergic potency, which has led to its use as a drug to alleviate pathological dry mouth symptoms (Bagheri et al., 1997). Atmoxetine is regarded as a highly selective drug with few side effects (Bymaster et al., 2002; Chamberlain et al., 2007). However, prolonged use of NE transporter blockers has been reported to increase the sensation of dry mouth and possibly also reduced salivation in approximately 20% of users (Samuels & Szabadi, 2008). These effects were observed after four weeks of daily doses of 60 mg/day to 120 mg/day. We are not aware of similar reports after acute

administration of a lower dose of atomoxetine, and our data provided little evidence of salivation effects. Therefore, confounding parasympathetic effects enhancing SAA secretion directly or via augmentation of peripheral sympathetic/noradrenergic effects, seem unlikely (cf. Bosch et al., 2011).

The absence of any increase in salivary flow rate in this study rules out an effect of cholinergic/parasympathetic nerves on SAA. This implies that increased central NE is mediating the increase in SAA through activation of the sympathetic nerves that innervate the saliva glands, which is supported by extensive neuro-anatomical data showing dense projections of relevant centers (e.g., locus coeruleus) onto pre-ganglionic sympathetic nuclei (Samuels & Szabadi, 2008).

4. Conclusions

This work points toward the potential value of SAA as an inexpensive and non-invasive procedure to obtain information about activation of the central NE system. The work implies that SAA may be used to mark endogenous fluctuations in central noradrenergic activation in a manner similar and complimentary to pupil dilation (c.f. Warren et al., 2016), thus providing a valuable tool for testing theories of the function and malfunction of the noradrenergic system. Further research, validating these findings in other contexts, and using other manipulations, possibly complimented with additional assays of glandular proteins sensitive to autonomic activity, (cf. Proctor & Carpenter, 2002), seems both warranted and necessary to bring this potential to fruition (Bosch et al., 2011; Nagy et al., 2015).

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Supplementary Material

Supplementary Methods: Exclusion criteria

Exclusion criteria included current use of prescription medication, a history of psychiatric illness, cardiovascular disease, renal failure, hepatic insufficiency, glaucoma, head trauma, hypertension, and drug or alcohol abuse. Participants with learning disabilities, poor eyesight (severe myopia of -6 diopters or worse), who smoked more than 5 cigarettes a day, or who were pregnant were also excluded.

Supplementary Results: *Flow Rate*

As shown in Figure S1, flow rate showed a small spike at T=105 minutes in the placebo condition that was not observed in the atomoxetine condition, yielding a marginally significant time by treatment interaction ($F(4,80) = 2.24$, $p = .071$). The effect of treatment was only significant at T=105, $t(23) = 3.29$, $p = .003$; at T=75 the effect was marginally significant, $t(23) = 1.86$, $p = .069$. It is interesting that in the placebo condition, our participants exhibited an acute increase in salivation at the sampling time following removal from an MRI scanner as a part of another experiment (van den Brink et al., 2016), and also, to a lesser degree, at the sampling time immediately preceding the scan. This spike in salivation was absent in the atomoxetine condition, suggesting that atomoxetine counteracted a parasympathetic response related to the MRI experience. This potential suppression of the parasympathetic response should not be considered an alternative explanation of our results. First, biochemically, suppression of salivation cannot be attributed to the action of another neuromodulatory system than NE. Second, mechanistically, the effect of atomoxetine on SAA was also observed when flow rate was controlled for using a measure of secretion rather than concentration.

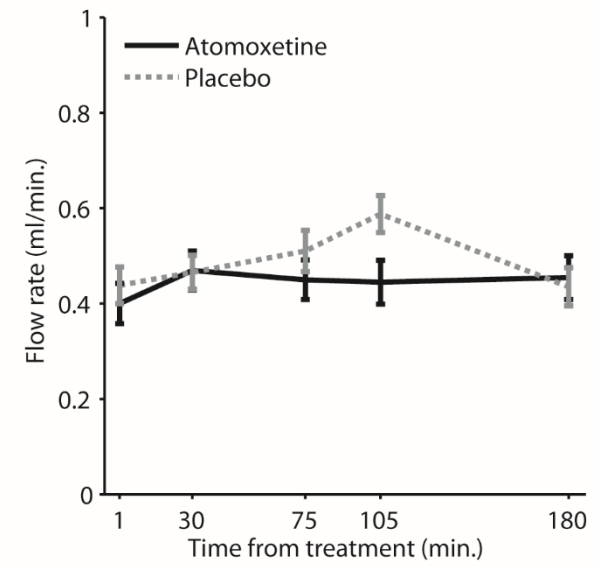

Figure S1. Effect of atomoxetine on salivary flow rate. Error bars reflect SEM.

Supplementary Results: Relationship between SAA and Cortisol

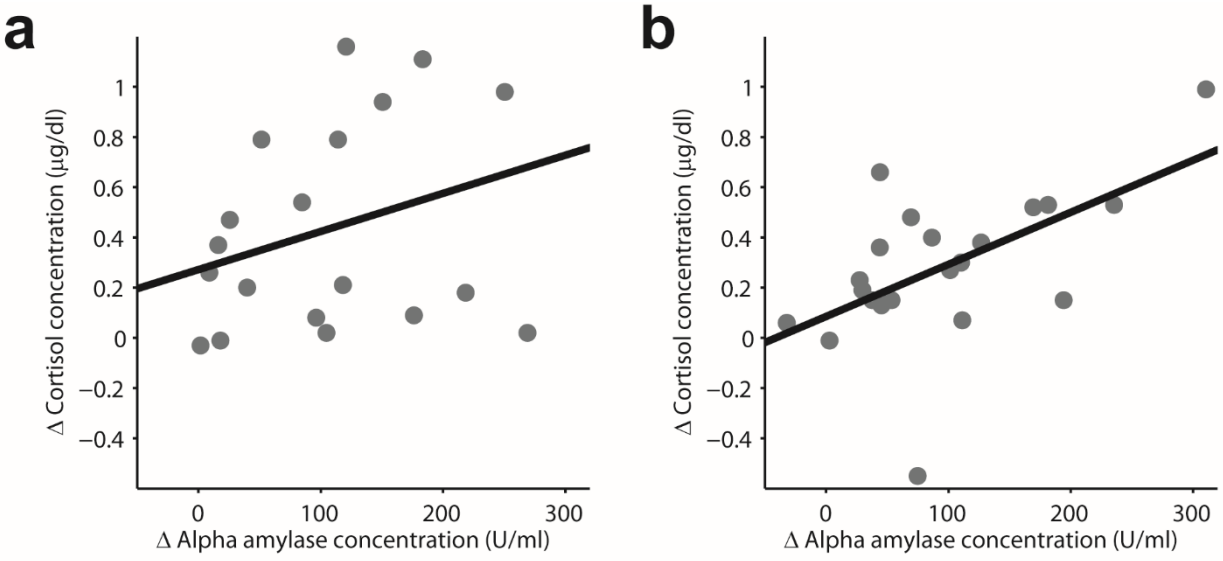

Figure S2. Relationship between drug effects on SAA and salivary cortisol. (a) Delta values were calculated by subtracting baseline from peak values after treatment. (b) Delta values were

calculated by subtracting mean concentrations from samples 3-5 in the placebo session from mean concentrations from samples 3-5 in the atomoxetine session.