

Effects of Muscimol in the Nucleus Accumbens Shell on Salt Appetite and Sucrose Intake: A
Microstructural Study with a Comment on the Sensitization of Salt Intake.

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Abstract

Previous work has demonstrated that injections of the GABA_A agonist muscimol into the nucleus accumbens shell (AcbSh) induce pronounced increases in the intake of solid foods and sucrose solutions, but do not potentiate water intake. In order to clarify the range of situations in which inactivation of the AcbSh potentiates ingestive behavior, we examined the effects of muscimol injections on the intake of a 3% NaCl solution in sodium-depleted animals. Although sodium-depleted subjects avidly consumed this solution, muscimol injections had no effect either on the volume consumed or on a variety of microstructural licking parameters. In contrast, in these same animals, muscimol injections significantly increased licking of a 10% sucrose solution. These results suggest that inactivation of the AcbSh may selectively increase the intake of foods, but not that of other homeostatically relevant ingestates. Examination of microstructural parameters suggested that the effect of muscimol on sucrose intake was not mediated by alterations in the “palatability” of the sucrose solution. We also observed that sodium-depleted subjects displayed significantly larger salt intakes after their second experience with sodium depletion than their first, and microstructural analysis in this case indicated that this sensitization effect was produced in a manner consistent with the animals showing increased “hedonic responsiveness” to the salt solution.

Keywords: Ventral striatum, Feeding, Licking, lickometer, Intracranial, Ingestive behavior, Sodium hunger, Sodium appetite, Sensitization

The nucleus accumbens (Acb) occupies a central role in contemporary theorizing about the neural basis of motivation, but the way in which this structure influences basic consummatory behaviors still remains unclear. A substantial number of studies have shown that GABAergic mechanisms within the medial nucleus accumbens shell (AcbSh) play an important role in the control of feeding. Large increases in the intake of both solid and liquid diets can be produced by injections of the GABA_A agonist muscimol into the AcbSh (Basso & Kelley, 1999; Reynolds & Berridge, 2002; Stratford, 2007; Stratford & Kelley, 1997; Stratford & Wirtshafter, 2011) and, conversely, blockade of GABA_A receptors at this site reduces food intake (Kandov et al, 2006; Stratford & Kelley, 1997). The effects of GABA agonists appear restricted to the medial AcbSh, and feeding cannot be induced by injections into the core or the lateral portions of the shell (Basso & Kelley, 1999; Reynolds & Berridge, 2001; Stratford & Kelley, 1997). Strikingly, however, inactivation of the AcbSh does not increase water intake (Basso & Kelley, 1999; Stratford et al, 1998; Stratford & Kelley, 1997; Stratford & Kelley, 1999; Stratford & Wirtshafter, 2004). The GABA_B agonist baclofen also increases intake of solid foods, but, again, does not alter water intake (Stratford & Kelley, 1997; Ward et al, 2000).

Given that many workers have suggested that the Acb plays a generalized role in basic processes such as reward or effort allocation (Carlezon & Thomas, 2009; Salamone et al, 2007; Sesack & Grace, 2010), it is surprising that inactivation of the AcbSh appears to affect the intake of foods, but not of water. It would seem of substantial theoretical importance to determine whether food intake is really uniquely affected by accumbens inactivation, or whether this procedure can also alter the ingestion of other motivationally relevant substances, such as salt solutions in sodium depleted animals. It has been known since the work of Richter (Richter, 1936) that sodium depletion induces pronounced intake of even concentrated salt and evidence

suggests that salt depletion selectively increases preferences for, and palatability of, sodium chloride solutions, as well as increasing the extent to which animals will work to obtain such solutions (Beauchamp et al, 1990; Berridge et al, 1984; Morris et al, 2008; Takamata et al, 1994). Substantial evidence also suggests that sodium depletion and subsequent salt ingestion can produce functional and anatomical alterations within the Acb (Loreaux et al., 2011; Lucas et al., 2000, 2003; Nair et al., 2007; Roitman et al., 2002; Voorhies & Bernstein, 2006), and anatomically related structures (Shekhtman et al, 2007; Tindell et al, 2009). This pattern of results suggests that the AcbSh might exert an influence over salt intake similar to that which it exerts over the intake of caloric foods. If inactivation of the AcbSh acts to potentiate ingestion of preferred, sapid, substances which act to relieve a physiological need, one would certainly expect that muscimol injections would increase salt intake in sodium deprived rats. Because of the theoretical importance of this issue, we here examined the effects of intra-AcbSh muscimol on intake of a 3% sodium chloride solution in rats treated with the diuretic agent furosemide. These studies were conducted using lickometers, and the patterns of licking were subjected to microstructural analysis which provides much more information about the details of ingestive behavior than can be obtained by simply looking at total intakes. We examined the effects of the intracranial injections using a repeated-measures, crossover design, in which each subject was tested after both saline and muscimol injections administered in a counterbalanced sequence. This procedure incidentally provided information about the microstructural effects of repeated salt depletion, a topic which does not appear to have been investigated previously. Finally, we examined the effects of intra-AcbSh muscimol injections on the microstructure of the intake of a 10% sucrose solution as a “positive control” in order to verify that AcbSh inactivation would indeed increase intake of a liquid food in these subjects

Method

Animals

Subjects were 36 male Sprague–Dawley rats obtained from Charles River (Chicago, IL) weighing at least 300 g at the time of surgery. Animals were individually housed in plastic cages with food and water available *ad libitum*, except as noted below.

Surgery

Rats were anesthetized with sodium pentobarbital (60 mg/kg) and 22-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted bilaterally using standard, flat-skull stereotaxic techniques. The guide cannulae were aimed to terminate 2.0 mm dorsal to the AcbSh at coordinates of anteroposterior: 1.6, mediolateral: ± 0.8 , and dorsoventral: -6.1 (mm from bregma). The guide cannulae were held in place by stainless steel screws and denture lining material and stainless steel obturators were inserted into the lumen of each cannula to help maintain patency. Rats were allowed to recover for at least seven days before testing began. Food and water were available *ad libitum*, except as noted below.

Apparatus

Testing was conducted in plastic chambers measuring 30 cm wide X 28 cm deep X 25 cm high with stainless steel bars forming the floor. The testing boxes were housed within sound attenuating chambers equipped with exhaust fans which provided ventilation and masking noise. A stainless steel drinking spout, attached to a 60 ml calibrated tube, was mounted 5 cm above the floor with the tip recessed 1 mm with respect to the with the inside of the chamber. Licks were detected with standard low current lickometer circuitry (<0.3 μ A current passed, according to the manufacturer, Med Associates, St. Albans, VT) and the time of individual licks recorded to the

nearest ms. Testing sessions lasted 60 min at which time the total fluid intake was measured and rats returned to their home cages.

Analysis of licking data.

Latency from placement in the testing apparatus to the first lick was measured as were the total number of licks made in each minute of the test session. Cumulative intake curves for each animal were constructed in one min time bins and fit, using a nonlinear least squares technique, to the exponential decay equation “cumulative licks= $ab(1-e^{-t/b})$ ” where “t” represents time in minutes, “a” represents the slope of the cumulative intake curve at $t=0$, (i.e., the estimated initial rate of licking), “b” represents the time constant of decay, and “e” the base of natural logarithms. Previous studies have shown that this equation provides excellent fits for cumulative intake curves (Davis & Levine, 1977; Davis & Perez, 1993; Genn et al, 2003; McCleery, 1977; Wirtshafter et al, 2011). Microstructural variables were defined as in previous studies by Davis and his coworkers (Breslin et al, 1996; Davis, 1989; Davis, 1998; Davis & Perez, 1993; Davis & Smith, 1992; Wirtshafter et al, 2011). Briefly, a cluster was defined as a string of at least three licks that was separated from other licks by an interval of more than 0.5 sec, and the number and mean size of clusters in each run was determined. (Some other authors have used a 1 sec cutoff (Spector et al, 1998), but the difference is of little practical significance, as only a very small proportion of interlick intervals (ILIs) occur in the range of 0.5 to 1.0 sec.). For reasons we have discussed in detail elsewhere, (Wirtshafter et al, 2011), burst size and number were not measured, but, in order to be consistent with the earlier literature, we have retained the term “inter-burst interval” (IBI) to refer to ILIs greater than 0.25 and less than or equal to 0.5 sec in duration. The proportion of all ILIs occurring in this IBI range was determined. Finally we use the term “within-burst ILIs” to refer to ILIs less than 0.25 sec in duration. Within-burst ILIs are

presumed to reflect the operation of a licking pattern generator and a great majority of ILIs fall in this range.

Sodium depletion

Animals were removed from their cages, weighed and then injected with the diuretic agent furosemide (Sigma Chemical Company, St. Louis) at a dose of 10 mg/kg, s.c. Fresh bedding was then placed in their cages, their standard tap water replaced by distilled water, and their standard lab chow by a sodium-deficient diet (Harlan, Madison, WI). Animals were tested 23 hr later, after which standard lab chow and tap water were returned.

Intracerebral injections

In order to make injections, rats were restrained gently, the obturators removed, and a 28-gauge stainless steel injection cannula, extending 2.0 mm beyond the tip of the guide, was inserted into each guide cannula. Rats then received simultaneous bilateral 0.50 μ l infusions at a rate of 0.33 μ l/min using a motor-driven microsyringe connected to the injection cannulae through a length of fluid-filled polyethylene tubing. The injection cannulae were left in place for an additional 60 s after the infusions in order to minimize leakage up the cannula track after which they were removed and replaced with the obturators. Animals were then returned to their home cages for a period of 10 min, to allow for drug diffusion, at which time they were placed in the operant boxes for their daily run. Each subject received one saline injection several days prior to the start of drug injections to acclimate them to the procedure.

Adaptation and testing procedure.

In order to accustom the animals to drinking in the test chambers, all subjects were given 5 days of pretraining under water deprivation. On each of these days, subjects were deprived of water for 23 hr before being placed in the test chambers for 60 min. Rats were then given a

further two hours to drink in their home cages after which water was again removed. On the first two of these days, water was available in the lickometer chambers and on the next three days, a 3% salt solution was presented. Animals were then taken off of the water deprivation schedule and placed in the lickometer chambers for 60 min tests for a further 5 days with only 3% sodium chloride available. Intakes were very low during this period. Rats were then given one day without placement in the lickometers, after which testing began.

All subjects at this time were subjected to sodium depletion, as described above, and intake of 3% NaCl was then measured 23 hours later in the lickometer chambers. Half of the subjects received intra-AcbSh injections of saline immediately before the tests, the other half received muscimol. Animals were not run on the following two days; on the two days after that they were again, in the nondepleted state, placed in the testing chambers with 3% NaCl available. They were not run on the following day, and on the day after that, they were again injected with furosemide and tested 23 hr later, one week after their initial drug session. Before this second test session, animals received the opposite injection to that they received following their initial depletion.

Following the completion of testing on the salt solutions, animals received seven daily placements in the lickometer chambers with a 10% sucrose solution available. The following day, half of the animals were injected with muscimol and half with saline before their test runs. Subjects were placed in the testing chambers in the absence of injections for the next two days and then received a final test session before which they were injected with the treatment complimentary to that they had received on their previous sucrose runs.

Statistical Analysis

In the sodium depletion phase of the experiment, all subjects were tested for salt intake after injections of both vehicle and muscimol which were administered in a counterbalanced order. In these types of situations, researchers typically tend to ignore the order of testing variable and simply compare experimental and control performance within individual subjects. Repeated sodium depletion is, however, known to affect subsequent intake (Falk, 1966; Sakai et al, 1987), and this effect would act to increase between-days variance which, if not accounted for, would make it more difficult to detect within subject changes in intake due to muscimol injections. This type of experimental situation, involving a so-called crossover trials design, has been dealt with extensively in the statistical literature and suitable analytic procedures have been developed. The most commonly recommended approach is to analyze the data using a split-plot analysis in which individual animals are considered as plots (Diaz-Uriarte, 2002; Jones & Kenward, 1989). This technique allows for an evaluation of the within subject effects of muscimol and of repeated depletion, but does not allow for an evaluation of a possible muscimol X repeated testing interaction, which would require a much more complex design. In order to rule out the remote possibility that a muscimol X repeated testing interaction might have occluded the detection of an effect present on the first testing day alone, we additionally conducted one way analyses of variance (ANOVAs) on data from just the first day, in which case muscimol would represent a between subjects effect. The results of these two different analyses were in agreement in every case. Data from the sucrose phase of the experiment were analyzed simply by one-way repeated-measures ANOVAs, as preliminary examination revealed no hint of an order effect.

Histology

Following the completion of behavioral testing, all subjects were perfused transcardially, under deep sodium pentobarbital anesthesia, with saline followed by 10% formalin. Several days later,

the brains were sectioned at a thickness of 60 μm , and the sections stained with cresyl violet to evaluate the injection site.

Blood Chemistry

In order to examine the effects of single and repeated sodium depletion on blood chemistry, three separate groups of 4 subjects each were subjected to either (1) two episodes of salt depletion separated by one week, using methods identical to those described above, (2) an injection of normal saline followed one week later by an injection of furosemide, or (3) two injections of normal saline. Twenty-three hours following the last injection, subjects were deeply anesthetized and samples of blood withdrawn from the left atrium into heparinized tubes. A complete blood count (CBC) was then performed on these samples using a standard clinical blood analyzer.

Results

Histology.

As is illustrated in Fig. 1, all cannula placements terminated within the ventral, medial portion of the AcbSh, at locations very similar to those we have examined in previous studies.

Behavioral Data

Salt intake - Macrostructure.

Intakes of the 3% salt solution following injections of saline or muscimol are shown in the upper left panel of Fig. 2, where it can be seen that furosemide-induced sodium depletion produced a robust consumption of the salt solution which tended to be larger after the second depletion than the first. Muscimol injections tended to slightly reduce intake as compared to the equivalent saline condition. Analysis of these results by means of a 2-way (muscimol X treatment day) split-plot ANOVA, as described in the methods section, indicated a significant

effect of test day ($F(1,22)=21.43$, $p<0.001$) but not of muscimol treatment ($p>0.1$). Analysis of total intakes on the first day of testing by means of a one-way ANOVA also failed to indicate a significant effect of muscimol treatment ($F<1$).

Total numbers of licks are shown in the upper right panel of Fig. 2, and a split-plot ANOVA conducted on these data indicated that more licks were made overall on the second treatment day than the first ($F(1,22)=19.8$, $p<0.001$), but that muscimol did not exert a significant effect ($F<1$). A one-way ANOVA conducted just on the data from the first day of testing again failed to detect an effect of muscimol. The number of licks required to consume a ml of fluid, a reciprocal measure of licking efficiency, is shown in the lower left panel of Fig. 2. Neither muscimol treatment nor day of testing significantly affected this variable ($F<1$ in both cases).

The lower right hand panel of Fig. 2 shows latencies from placement in the lickometer chambers until the start of licking. The split-plot ANOVA indicated that latencies were significantly shorter on the second day of testing than the first ($F(1,22)=10.46$, $p<0.005$), but that there was no consistent effect of muscimol ($F<1$). Individual ANOVAs also indicated that the muscimol effect was not significant on either of the two days of testing ($p>0.2$).

Numbers of licks per min. across the 60 min test session are shown in Fig.3, which illustrates that rate of licking in the initial parts of the test sessions tended to be higher on the second than the first day of testing. In order to analyze the time course of licking in more detail, the cumulative intake curves for individual animals were fit, as described in the methods section, to the exponential decay equation “cumulative licks= $ab(1-e^{-t/b})$ ” where “t” represents time in minutes, “a” represents the slope of the cumulative intake curve at $t=0$, (i.e., the estimated initial rate of licking), “b” represents the time constant of decay, and “e” the base of natural logarithms. The mean r^2 value for goodness of fit was 0.961. Mean values for the “a” and “b” parameters and

typical fits are shown in Fig. 4. The lower left panel shows that values of the initial rate parameter tended to be higher on the second day of testing than the first, a result which was supported by the split plot ANOVA ($F(1,22)=9.70$, $p<0.005$). In contrast, muscimol tended to increase the “a” parameter on the first day of testing, but decrease it on the second. The overall effect of muscimol was not significant ($F<1$), and separate one-way ANOVAs indicated that the muscimol effect was not significant on either the first ($p>0.3$) or second day, although there was a trend in the latter case ($p<0.1$). Data for time constants (“b”) are shown in the lower right hand panel of Fig. 4 where it can be seen that neither day nor muscimol exerted a significant effect ($F<1$ in both cases). The mean meal duration (log transformed), i.e., the time interval from the first to the last lick, was significantly longer on the second day of testing than the first ($F(1,22)=5.23$, $p<0.01$), but was not altered by muscimol (data not shown)

Salt Intake – Microstructure.

Microstructural data are shown in Fig. 5. As can be seen in the upper panels of the figure, within burst ILIs (i.e., the mean of all inter-lick intervals less than 0.25 sec) were almost identical in all four test conditions ($F<1$ for both effects) as were the proportion of ILIs falling in the IBI range, that is, between 0.25 and 0.5 sec ($F<1$). The lower left panel shows that the number of clusters, that is groups of licks separated from each other by intervals of more than 0.5 sec, were unaffected either by day of testing or by muscimol injections. In contrast, the lower right panel of Fig. 5 shows that mean cluster size was significantly larger on the second day of testing than the first ($F(1,22)=25.26$, $p<0.001$), but that drug treatment was without significant effect ($F<1$).

Sucrose Intake

Of the original 24 rats, two lost their cannulae and one became ill before the completion of sucrose testing, so data for this portion of the experiments are available for only 21 subjects. Due

to a procedural error, absolute fluid intakes were not recorded from the animals in this phase of the experiment, but licking data are available for analysis and the results are summarized in Table 1. Muscimol injections produced a significant increase in the number of licks of the 10% sucrose solution ($F(1,20)=16.224$, $p<0.001$) and a significant reduction in the latency to initiate licking ($F(1,20)=8.04$, $p\leq 0.01$). Analysis of exponential curve fits showed that muscimol produced a small but significant reduction in the initial rate parameter “a” ($F(1,20)=5.87$, $p<0.025$), but produced a large increase in the time constant “b” ($F(1,20)=22.49$, $p<0.001$). Mean meal durations (log transformed) were significantly increased by muscimol treatment ($F(1,20)=15.3$, $p<0.001$). The total number of clusters generated was significantly increased by muscimol ($F(1,20)=13.14$, $p<0.002$), but mean cluster size was almost identical in saline and muscimol treated animals ($F<1$). As was the case for animals consuming sodium chloride, muscimol injections failed to alter the within burst licking rate of animals consuming sucrose ($F<1$) but did significantly increase the proportion of licks falling in IBI range, between 0.25 and 0.5 sec ($F(1,20)=5.50$, $p<0.05$).

Blood Chemistry

The upper panel of Fig. 6 shows blood hemoglobin concentrations in control animals and in subjects injected with furosemide either for the first or second time. Analysis of these data by a planned comparisons ANOVA indicated that furosemide significantly increased blood hemoglobin concentrations ($F(1,9)=6.87$, $p<0.03$), but that the magnitude of the effect did not differ in animals depleted one or two times ($F<1$). In contrast, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were almost identical in the three treatment groups (not shown). Red blood cell counts, shown in the lower panel of Fig. 6, were also significantly elevated by furosemide with respect to saline injections ($F(1,9)=14.37$,

$p < .005$)), but again there was no effect of previous depletions ($F < 1$). As would be expected given these results, an identical pattern was obtained with respect to hematocrit which was again increased to a similar extent by one or two furosemide injections. (not shown). No other CBC parameters were significantly altered by furosemide.

Discussion

The principal finding of the current study is that injections of muscimol into the AcbSh did not increase licking of a sodium chloride solutions in sodium-depleted animals. These results extend those of previous studies conducted in sodium-replete subjects (Basso & Kelley, 1999), a condition under which the preference for, and motivation to obtain, salt solutions is much lower than following sodium depletion. The fact that overall salt intake significantly increased the second time animals were depleted indicates that our failure to observe an effect of muscimol injections on the first test day cannot have been due to a ceiling effect, and further suggests that inactivation of the AcbSh does not produce effects similar to those seen after sensitization of salt appetite. Muscimol injections not only failed to alter the volume of the salt solution which was drunk, and the number of licks made, but were also without significant effect on a number of other measures of drinking behavior including the latency to initiate licking, the initial rate of licking and the time course with which it decayed, cluster size, number of clusters and the frequency of inter-lick intervals between 0.25 and 0.50 sec (IBIs). Equivalent results were obtained whether we analyzed data just from the first salt depletion test, using between subject statistics, or examined the results from both depletion periods using the within-subject split plot analysis. In contrast, we were able, in these same subjects, to confirm prior observations that muscimol infusions into the AcbSh increase licking for sucrose solutions (Basso & Kelley, 1999; Stratford & Wirtshafter, 2007, 2011). These findings demonstrate that the animals studied here

indeed had cannulae placed at sites from which certain ingestive behaviors can be potentiated and suggest that, in contrast to its dramatic effects on the intake of a number of caloric substances, inactivation of the AcbSh does not increase the intake of salt solutions even under conditions where they are ingested avidly. In a similar fashion, AcbSh inactivation does not alter intake of water in either deprived or nondeprived animals (Stratford & Kelley, 1997; Stratford et al., 1998; Stratford & Wirtshafter, 2004). Clearly any theory of the influence of the AcbSh on ingestive behavior must account for the effect that inactivation of this structure stimulates the intakes of some but not all substances.

A substantial proportion of Acb neurons are inhibited during ingestive behavior or in response to the infusion of various palatable substances into the oral cavity (Krause et al, 2010; Nicola et al, 2004; Roitman et al, 2005). It is especially striking that a similar predominance of inhibitory responses has been reported following intra-oral infusions of either sucrose or, in sodium-depleted animals, of salt solutions (Loriaux et al, 2011; Roitman et al, 2005). It has further been shown that individual cells tend to respond in similar ways to the presentation of different ingestates (Carelli et al, 2000; Roop et al, 2002). It has been suggested that these neurons may act to inhibit ingestive behaviors (Krause et al, 2010; Nicola et al, 2004), a conclusion consistent with the ability of electrical stimulation of the Acb to induce pauses in ongoing licking (Krause et al, 2010). It seems plausible that the ability of intra-AcbSh infusions to promote consummatory behavior might result from the pharmacological inhibition of these types of cells. Several authors have suggested, for example, that inactivation of the AcbSh may disinhibit motor program generators with the effect that ingestive behavior is released (Baldo & Kelley, 2007; Kelley et al, 2005; Krause et al, 2010; Meredith et al, 2008), a notion which seems compatible with the well known suggestion of Mogenson and his colleagues (Mogenson et al, 1980) that the

accumbens serves to link limbic and motor systems. Certain aspects of the current results would, however, appear to pose difficulties for these theories. For example, infusions of muscimol did not alter cluster sizes in rats consuming either saline or sucrose, as might have been expected if AcbSh inactivation blocked mechanisms which normally act to induce pauses in licking. Additionally, the fact that muscimol increases the intake of some, but not all, fluids seems to pose serious problems for any theory which proposes that the effects of AcbSh inactivation are mediated through disinhibition of motor mechanisms. If the same motor mechanisms are involved in the licking of sucrose solutions, salt solutions and water, why does intra-AcbSh muscimol only increase intake of the first of these fluids?

The contrast between the broad tuning of many accumbens cells, which respond to multiple natural reinforcers, and the highly restricted nature of the ingestive effects produced by inactivation of the AcbSh, which currently appear limited to the intake of foodstuffs, is striking. Three sorts of accounts of this discrepancy are possible. First, muscimol in the AcbSh may induce feeding by inhibiting a different population of cells than those which are suppressed during the consumption of multiple ingestates. A substantial proportion of accumbens cells are not responsive to acute intra-oral infusions and are not inhibited during consummatory behavior (Carelli et al, 2000; Krause et al, 2010; Loriaux et al, 2011; Roitman et al, 2005; Roop et al, 2002). If inactivation of some of these cells were responsible for the induction of feeding, the apparent lack of specificity seen in other accumbens neurons would be irrelevant, since these cells would not be the ones underlying the feeding effect. It is possible, for example, that the activity of the relevant cell populations might normally be modulated by stimuli arising from deprivation (Timofeeva & Richard, 2001) or from learned cues related to feeding (Park & Carr, 1998), rather than by the acute presence of intra-oral stimuli. Additionally, it is notable in this

regard that cells inhibited during ingestion are found not only in the AcbSh, but also in a number of adjacent regions including the accumbens core, the olfactory tubercle, and the dorsal striatum (Krause et al, 2010), none of which support feeding in response to muscimol injections.

A second possibility is that even though Acb cells respond in a similar way to a number of different reinforcers, the activity in these cells may preferentially influence feeding. Rather than acting directly on motor mechanisms, cells in the AcbSh may primarily influence modulatory circuitry which specifically affects feeding behavior. For example, the lateral hypothalamus (LH) plays an essential role in mediating the feeding induced from inactivation of the AcbSh (Baldo et al, 2004; Maldonado-Irizarry et al, 1995; Stratford, 2005; Stratford & Kelley, 1999; Stratford & Wirtshafter, 2012) and excitation of LH cells by means of local injections of excitatory amino acid agonists increases food, but not water, intake (Duva et al, 2002; Stanley et al, 1993). Similarly, glutamate release from the LH is increased in animals eating solid foods or drinking milk, but not in subjects who drink water (Thongkhao-on et al, 2008). If AcbSh efferents inhibited by muscimol were to terminate on these feeding specific mechanisms, it would be expected that their effects on consummatory behaviors would be limited to feeding. Much less is known about the neural substrates of salt ingestion, but the available data again demonstrate that certain brain systems may have very different effects on food and salt intake. For example, neuropeptide Y (NPY), which plays an essential role in mediating muscimol-induced feeding (Stratford & Wirtshafter, 2004) may actually reduce the intake of salt solutions in depleted animals (Madden et al, 1999).

A third possibility is that the apparent discrepancies between the effects of AcbSh inactivation on food, salt, and water intake may not reflect intrinsic differences between the various motivational systems, but may instead be a result of behavioral differences produced by the

specific deprivation parameters or the palatability or other properties of tastants which have been examined to date. A great deal of further work will be needed to conclusively evaluate this possibility, but it should be noted that effects on food intake can be observed under a number of different experimental conditions which are associated with marked differences in the vigor of the consummatory behavior. For example, muscimol increased the intake of a 10% sucrose solution which, after control injections, is consumed in large amounts with larger cluster sizes and higher initial rates than seen with the salt solutions examined here. In other studies, however, (in preparation) we have observed that muscimol injections also produce robust increases in the intakes of corn oil solutions, which are normally consumed only in small quantities with cluster sizes and initial rates lower than those seen here with salt. Muscimol injections also robustly increase intake of lab chow, which is only eaten in very small amounts under control conditions (Stratford & Kelley, 1997; Stratford & Wirtshafter, 2004, 2011). These findings suggest that there is not a simple relationship between the vigor of responding under baseline conditions and the magnitude of the response to muscimol.

Although intended primarily as a “positive control,” our examination of effects on sucrose intake also provided novel information about the microstructural basis of muscimol’s actions. The failure of muscimol to increase mean cluster size, or the initial rate parameter (“a”), suggests that its ability to increase licking for sucrose is not due to changes in the “perceived palatability” of the ingestate (Davis, 1989, 1998; Davis & Levine, 1977; Davis & Perez, 1993; Davis & Perez, 1993; Davis and Smith, 1992; Wirtshafter et al, 2011), a conclusion consistent with that of other workers employing different methodology (Reynolds & Berridge, 2002). The effect of these injections was due primarily to an increase in the number of clusters and the time constant (“b”) of the exponential curve fits. This pattern of results could reflect insensitivity to postingestive

feedback, although other types of effects might produce similar changes (Wirtshafter et al, 2011). Muscimol injections also significantly reduced latency to initiate licking of the sucrose solution, suggesting, as have other studies (Stratford & Wirtshafter, 2012; Wirtshafter & Stratford, 2010), that insensitivity to feedback cannot be the only alteration produced by AcbSh inactivation.

Subjects in the current study were tested twice following sodium depletion and displayed significantly larger intakes, and significantly shorter latencies to initiate consumption, on their second opportunity than on their first. These results replicate the well known phenomenon of sensitization of salt appetite (Falk, 1966; Sakai et al, 1987). In contrast to these behavioral differences, alterations in blood chemistry, reflecting alterations in plasma volume, were equivalent in animals depleted for the first or second time. In agreement with the results of previous investigators (Na et al., 2007; Sakai et al, 1987), these findings suggest that sensitization of salt appetite does not result from alterations in the ability of repeated treatments to deplete sodium.

Although it was not the major goal of our study, the current results provide, to our knowledge, the first microstructural analysis of the events underlying sensitization of salt appetite. Strikingly, sensitization appeared to exert its major effect on the initial rate parameter (“a”) and on cluster size, both variables which have been taken to reflect the “palatability” of the ingestate. In contrast, the time constant “b” did not change as a function of repeated testing, the longer **episodes of ingestion** seen on the second test day being the result of the animals starting from a higher initial rate of licking from which they decayed at the same rate as they had on the first day. These results suggest that prior experiences with depletion act to increase the “hedonic responsiveness” of animals to salt solutions. This result is surprising since previous studies (Clark & Bernstein, 2006) have found that sensitization does not alter orofacial responsiveness to

intra-oral infusions of salt solutions, which has often been taken to be another measure of “palatability”. Two principle explanations of this discrepancy are possible: First, subjects in the current study had the opportunity to consume a salt solution during their first depletion experience. Although experience with consumption under depletion is not essential for sensitization of salt appetite as measured in terms of intake or willingness to work (Falk, 1966; Sakai et al, 1987), it is possible that it plays an essential role in the development of alterations in hedonic responsiveness. Some evidence suggests, for example, that flavor preferences can be conditioned in humans to fluids paired with sodium chloride administration following exercise-induced salt loss (Wald & Leshem, 2003). Secondly, it is possible that orofacial reactivity during intraoral infusions and cluster size during spontaneous licking do not provide measures of the same underlying phenomenon. It may be that factors other than “palatability” can influence one or the other of these measures or that “palatability” itself may not represent a single, unitary, process. Further work will be required to determine the correct explanation for the differential effects of salt sensitization on these two measures of ingestive behavior, and such investigation would seem likely to yield important insights into the mechanisms underlying ingestive behavior.

In conclusion, the current experiments demonstrate that inactivation of the AcbSh has little effects on the intake of a salt solution, even in sodium-depleted animals. These results lend further support to the view that muscimol injections in the AcbSh have relatively specific effects on the intake of caloric substances. A major question is how the apparently specific nature of these effects can be integrated into general theories of the function of the nucleus accumbens.

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Figure Captions.

Fig. 1. Schematic representation of the location of cannula tips within the AcbSh; all tips terminated within the shaded region. LS: lateral septum, AcbC: nucleus accumbens core, CPu: caudate-putamen. Section modified from (Paxinos & Watson, 2007)

Fig. 2. Effects of intra-AcbSh injections of saline and muscimol on salt intake by sodium-depleted animals on the two days of testing using a crossover-trials design. Note that the subjects who received saline on the first test day received muscimol on the second, and *vice versa*. Panel A. Total intake across the 60 min test sessions. Panel B. Total numbers of licks emitted across the test session. Panel C. Mean numbers of licks needed to consume 1 ml of fluid. Panel D. Mean latency from placement in the test chamber to the first lick. Error bars indicate S.E.M.s. *=significant overall difference between the first and second days of testing ($p < 0.005$).

Fig. 3. Temporal course of licking of a 3% NaCl solution by subjects who received intra-AcbSh injections of either saline or muscimol before either their first or second experience with drinking under salt deprivation.

Fig. 4. The upper two panels show examples of fits of cumulative licking curves for individual sodium-deprived animals to the equation “cumulative licks= $ab(1-e^{-t/b})$ ” (see text for details) after injections of saline (filled symbols) or muscimol (unfilled symbols). Panel A is from a subject who received saline on his first day of testing and muscimol on his second, and Panel B from a

subject who received these treatments in the reversed order. Values for the “a” and “b” parameters under each condition are indicated in the figures and it can be seen that the differences between the curves are primarily due to increase in the initial rate parameter (“a”) on the second day of testing. Panels C and D show the mean values of the initial rate parameter (“a”) and time constant (“b”) after saline or muscimol injections on the two days of salt deprivation. Note that the subjects who received saline on the first test day received muscimol on the second, and *vice versa*. Error bars indicate S.E.M.s. *=significant overall difference between the first and second days of testing ($p<0.005$).

Fig. 5. Microstructural aspects of licking behavior for rats consuming 3% NaCl solution following intra-AcbSh injections of saline or muscimol on either their first or second experience with salt deprivation. Note that the subjects who received saline on the first test day received muscimol on the second, and *vice versa*. Panel A shows mean within-burst interlick intervals (ILIs), that is the mean of all ILI’s less than 0.25 sec in duration. Panel B shows the per cent of ILIs falling in the IBI range (between 0.25 and 0.50 sec). Panel C shows mean numbers of clusters of licks (i.e., groups of at least three licks separated from other licks by intervals of greater than 0.5 sec) produced across the test session, and Panel D shows mean cluster sizes. Error bars indicate S.E.M.s. *=significant overall difference between the first and second days of testing ($p<0.001$).

Fig. 6. Blood hemoglobin concentration (upper panel) and red blood cell count (lower panel) of rats injected two times with saline, once with saline and a second time with furosemide, or twice

with furosemide. The two injections were separated from each other by one week, and the animals were sacrificed 23 hours after the second injection.

Figure 1

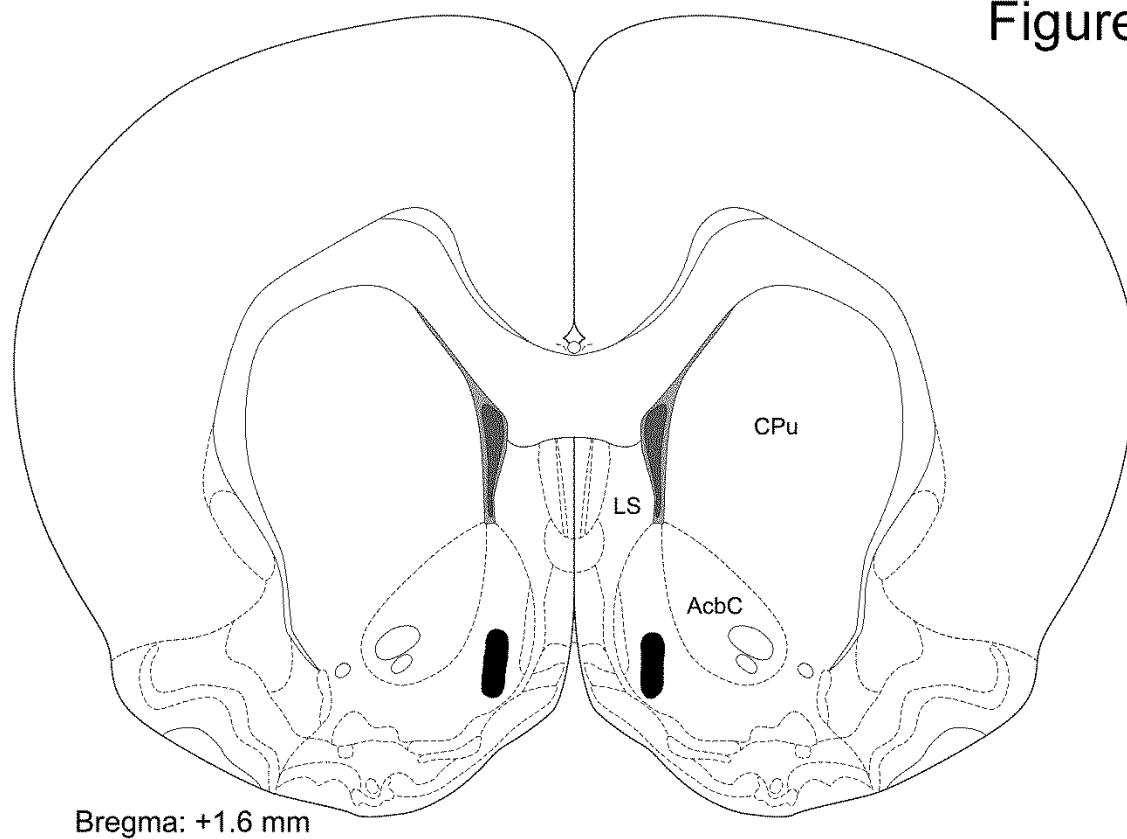


Figure 2

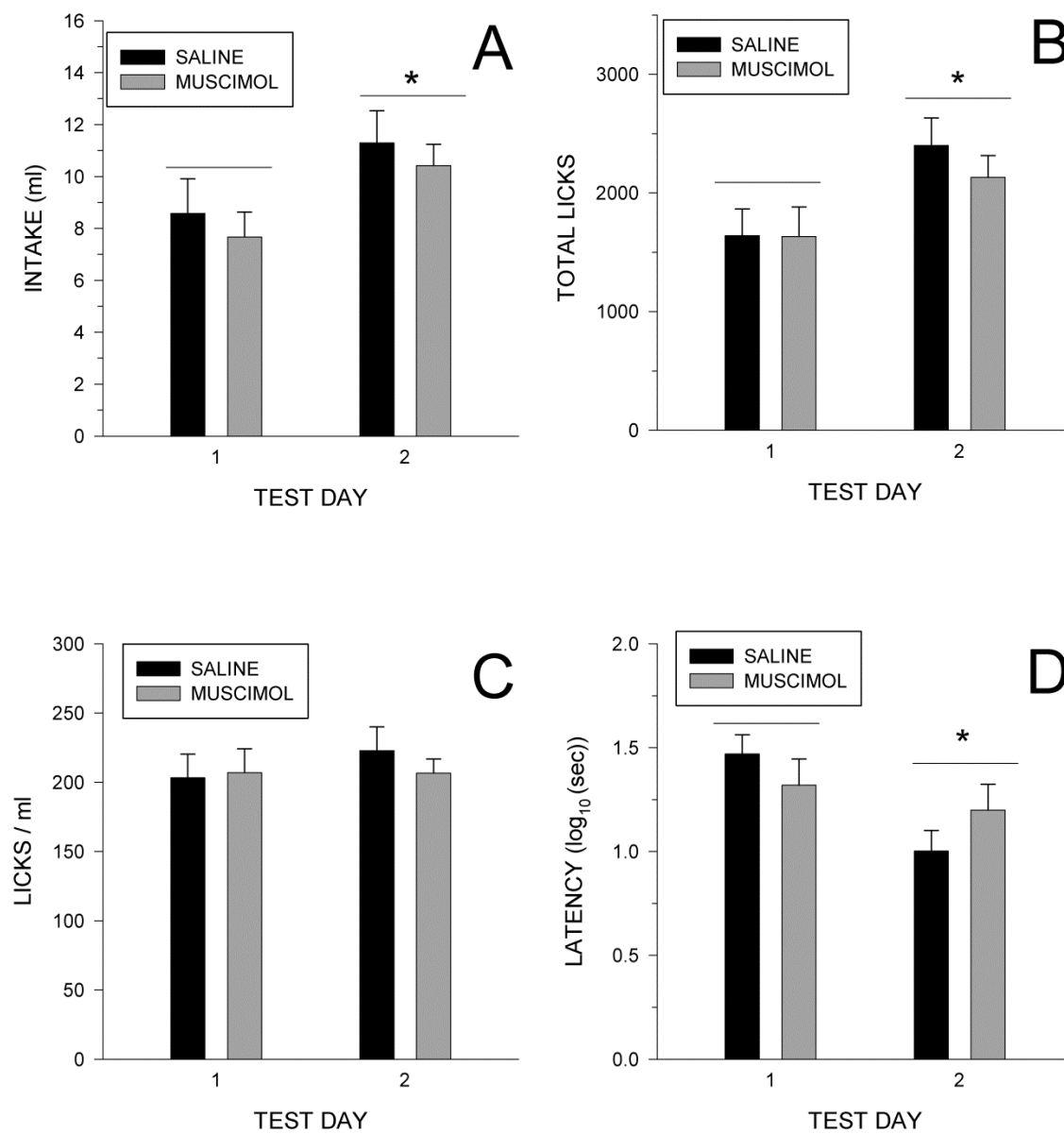


Figure 3

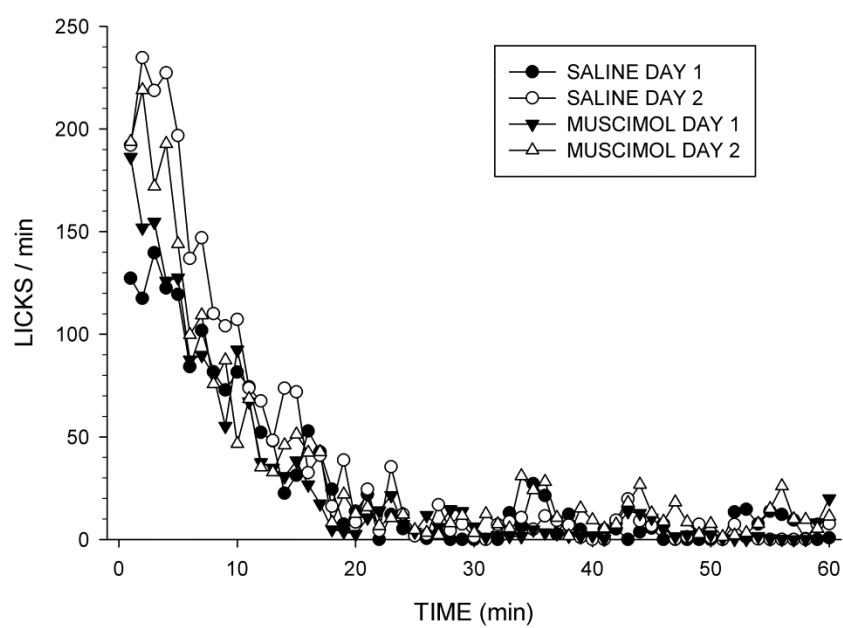


Figure 4

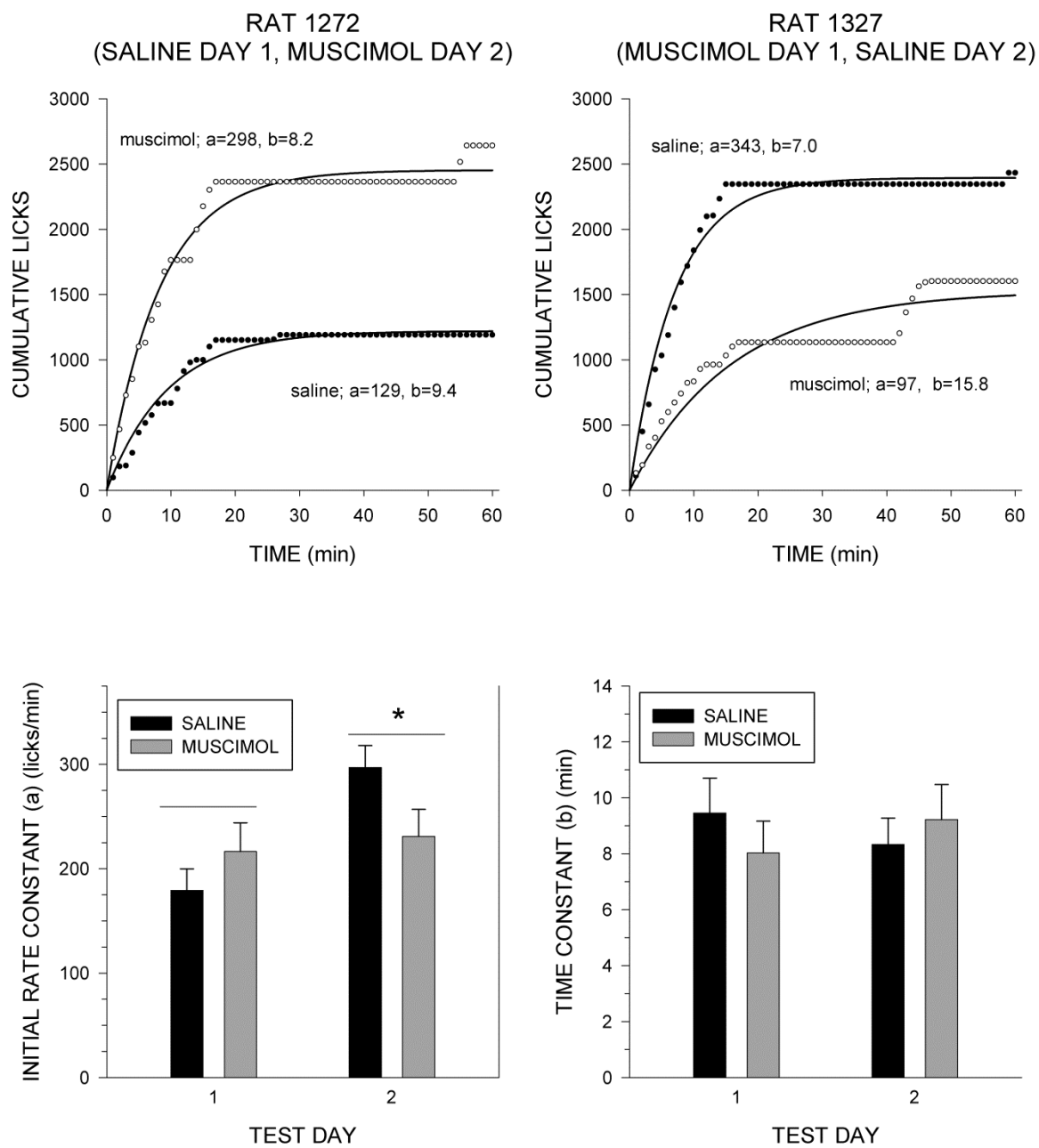


Figure 5

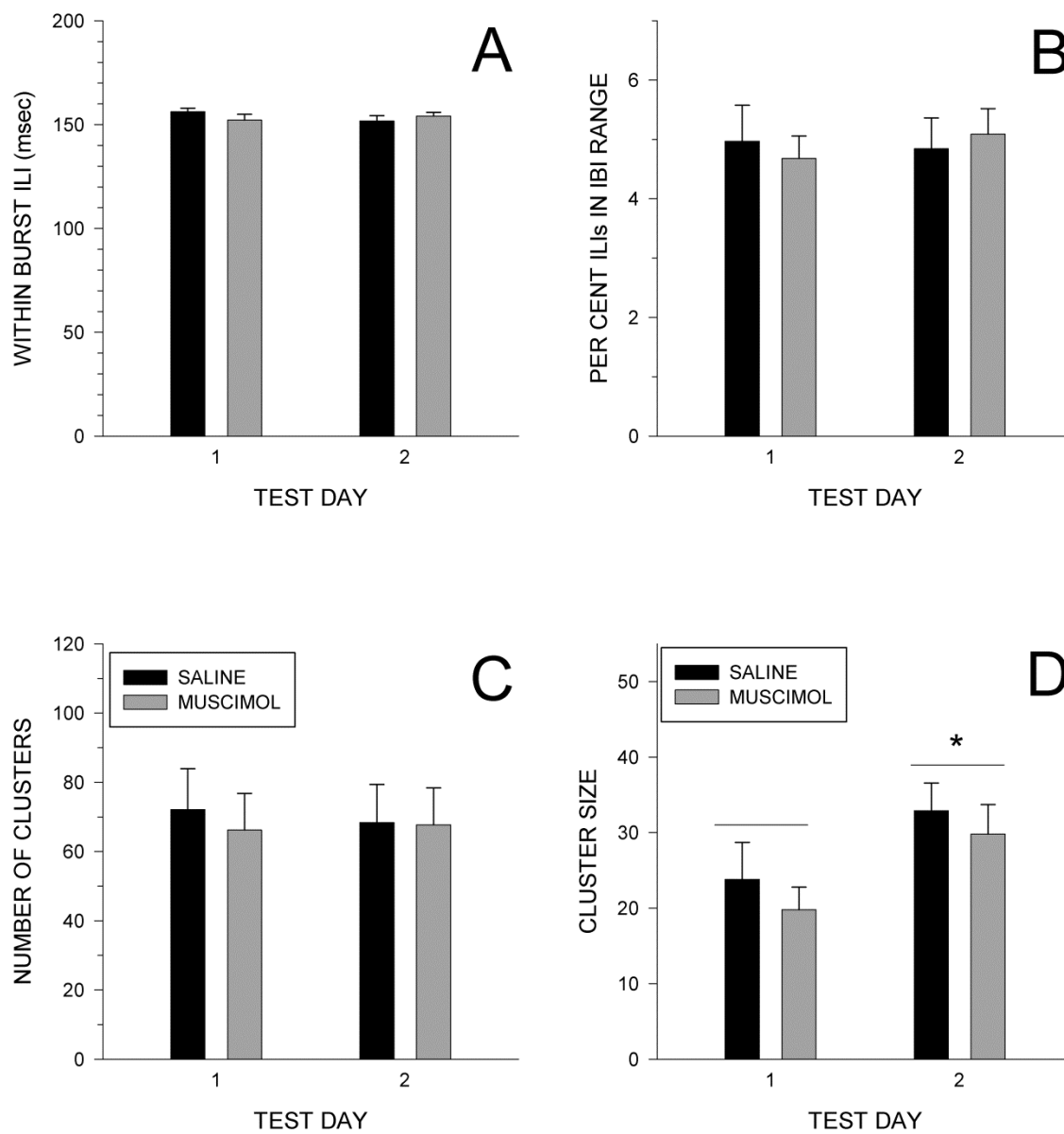


Figure 6

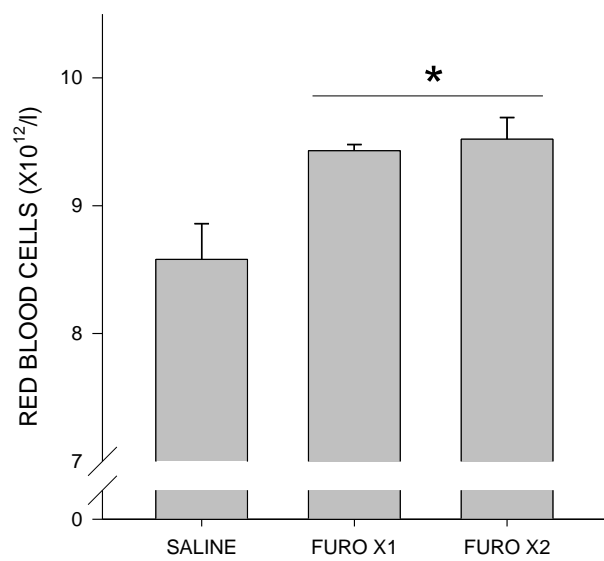
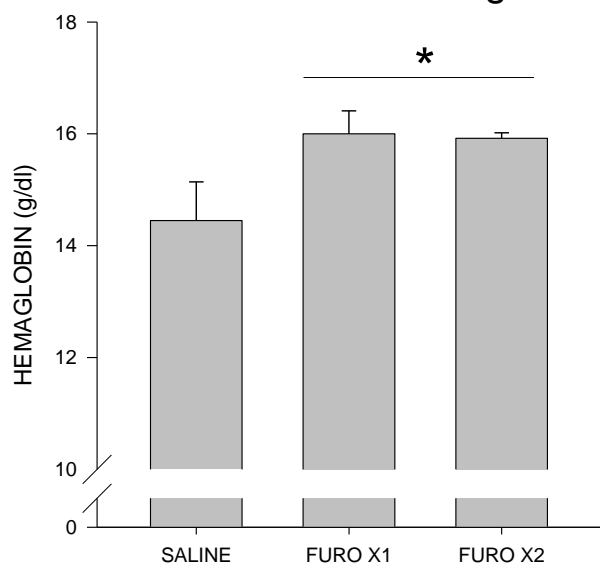


Table 1
Effects of intra-AcbSh muscimol on sucrose intake.

	Saline	Muscimol
Total licks	2710.0 ±220.2	4018.0*** ±305.1
Latency (sec)	28.1 ±6.3	12.2** ±3.8
Initial rate parameter (a)	368.1 ±13.9	311.8* ±23.5
Time constant (b)	7.1 ±0.5	14.3*** ±1.4
Meal duration (sec)	2007.4 ±258.6	3041.6*** ±210.6
Cluster number	35.4 ±6.7	49.0** ±6.9
Cluster size	84.6 ±7.3	79.3 ±8.3
Within-burst interlick interval (msec)	14.9 ±0.2	15.0 ±0.2
Per cent ILIs in IBI range	2.6 ±0.4	3.4* ±0.5

*=p<0.05, **=p<0.01, ***=p<0.001