Taste discriminating capability of different bitter compounds by the larval styloconic sensilla in the insect herbivore *Papilio hospiton* (Géné).

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Abstract

Herbivorous animals may benefit from the taste discriminating capability of bitter compounds because plants produce noxious compounds, some of which are toxic while others are only unpalatable. Our goal was to investigate the contribution of the peripheral taste system in the discriminating process of different bitter compounds by an herbivorous insect using the larvae of *Papilio hospiton* Géné as experimental model, showing a narrow choice range of host plants. The spike activity from the lateral and medial styloconic sensilla, housing two and one bitter-sensitive gustatory receptor neurons (GRNs) respectively, was recorded following stimulation with nicotine, caffeine, salicin and quercitrin and the time course of the discharges was analyzed. Nicotine and caffeine activated all three bitter-sensitive GRNs, while salicin and quercitrin only two of them. In feeding behaviour bioassays intact larvae ate glass-fiber disks moistened with salicin and quercitrin, but rejected those with nicotine and caffeine, while lateral sensillum-ablated insects also ate the disks with the two latter compounds. The discriminating capability among bitter taste stimuli and the neural codes involved in the larvae of *P. hospiton* are discussed.

Key Words: chemoreception, bitter discrimination, lepidopterous larvae, feeding behaviour, Papilionidae.

1. Introduction

The taste system of all animals has separate gustatory receptors directed to recognize different taste modalities, such as cells responding selectively to sugars (phagostimulants) and others to compounds that humans taste bitter (deterrents), evoking an appetitive or aversive behaviour to edible or noxious foods, respectively (Kvello et al., 2010). This organization allows to distinguish among compounds of different taste modalities, but may limit the ability to discriminate among chemicals that activate the same gustatory receptors (Masek and Scott, 2010). The ability to discriminate among different bitter compounds is particularly useful for herbivorous animals. In fact, plants contain secondary metabolites that humans taste bitter. Some of these compounds are toxic, others are harmless (Bate-Smith, 1972; Brieskorn, 1990; Brower, 1984; Garcia and Hankins, 1975; Glendinning, 1994).

Which neural code is used to identify and discriminate the taste stimuli both among modalities and within a same modality, is still a matter of debate (Caicedo et al., 2002; Liman et al., 2014).

Two different taste coding models are generally proposed to mediate taste detection and subsequent behaviour (Dethier and Crnjar, 1982; Dethier, 1993; Glendinning et al., 2006; Marella et al., 2006; Smith and John, 1999; Yarmolinsky et al., 2009). According to the "labeled-line" model (LL), gustatory receptor neurons (GRNs) are addressed specifically to respond to different ligands and their activation is wired to specific behavioural outputs (Marella et al., 2006; Liman et al., 2014). This model is supported by the observation that some GRNs are activated by sugars and low levels of fatty acids, both promoting feeding (Wisotsky et al., 2011), while others are activated by bitter compounds and high concentrations of salt, which suppress feeding (Hiroi et al., 2004). In addition, a subgroup of bitter-sensitive GRNs are also activated by low pH levels of carboxylic acids, which likewise deter feeding (Charlu et al., 2013). Instead, the "across neuron pattern" model (ANP) assumes that GRNs respond to different qualities so that the combination of different modalities of activation can evoke specific behaviours (Caicedo et al., 2002; Dethier and Crnjar, 1982). However, other neural coding paradigms seem to contribute

to the discrimination process of the gustatory information: the number of action potentials evoked (rate or frequency code), relative pattern of activity of each GRN activated (ensemble code), the discharge pattern (temporal code) and temporally dynamic ensemble codes (spatio-temporal code) (Dethier and Crnjar, 1982; Glendinning et al., 2006; Weiss et al., 2011).

While it is generally accepted that animals are able to discriminate between different taste modalities, little is known about their ability to discriminate between compounds which evoke the same feeding behaviour. Recent studies conducted by Masek and Scott (2010) argue that the taste system of *Drosophila melanogaster* is not able to discriminate between different sugars or different bitter compounds, while as reported by Weiss et al. (2011), the bitter-sensitive GRNs respond differently to various bitter substances in terms of temporal dynamics of receptor activation and specificity of response. The latter results are also in agreement with those reported in *Manduca sexta*, in *Pieris sp*. larvae (Glendinning et al., 2006; van Loon and Schoonhoven, 1999) and in mammals (Di Lorenzo et al., 2009; Wilson et al., 2012).

Lepidopterous larvae are a simple and attractive model for studying the neural coding mechanisms of taste information because they have a few GRNs, the dendrites of which interact with taste stimuli in the environment, and the axons of these neurons project directly, without synapsing, to a region of the brain called subesophageal ganglion (SOG). Host specificity of lepidopteran insects is determined not only by female oviposition preferences, but also by larval food acceptance. In some cases, larvae may have no choice and need to adapt to the plant where they hatched. In this respect we considered that the discriminating capability of the larval peripheral taste system plays an important role in feeding acceptance governed by the ability to discern between noxious and harmless secondary metabolites.

Our experimental model, the larvae of *Papilio hospiton* Géné, is an oligophagous species using a few host plants in the Apiaceae and Rutaceae families. However, in Sardinia, *P. hospiton* can be considered practically monophagous using the giant fennel (*Ferula communis* L.) as an almost

exclusive host plant: only if *Ferula* is unavailable two other plants are used, one narrow endemic (*Ferula arrigonii* Bocchieri*)* and the other rare (*Ruta lamarmorae* Bacch., Brullo & Giusso) (unpublished data).

Aim of this study was to evaluate the discriminating capability among different bitter compounds and which neural code/s is/are used in the peripheral taste system of *P. hospiton* larvae, by means of an electrophysiological and behavioural approach. Spikes activity was recorded from the lateral and medial maxillary styloconic sensilla for two reasons. First, in a previous study, we found that styloconic sensilla of each maxilla house three bitter-sensitive GRNs: two in the lateral and one in the medial sensillum (Sollai et al., 2014). Second, even if styloconic sensilla are not the only sensilla in the peripheral taste system of lepidopterous larve, their GRNs appear to play a major role in the recognition of plants food (Schoonhoven and van Loon, 2002).

2. Materials & Methods

2.1 Insects and rearing

Papilio hospiton Géné larvae were obtained from eggs laid in the butterfly oviposition annex (a 3 x 3 x 3m cage) of the Physiology laboratories (University of Cagliari) by lab stock adult females on potted giant fennel (*Ferula communis* L.). Caterpillars were reared at the insectary annex of the Physiology laboratories (University of Cagliari) in 1500-ml plastic cups (4-5 per cup) kept in an environmental growth chamber (24-25 °C, 70% R.H., 16 h light/8 h dark photoperiodic regime) and checked daily until fit for the experiments.

Fresh foliage of *F. communis*, came from plants grown in a yard adjacent to the butterfly cage, was provided everyday and was available ad libitum.

2.2 Electrophysiological experiments

Electrophysiological recordings were obtained from 5th instar larvae two days after moulting (Simmonds et al., 1991) from the medial and lateral maxillary styloconic sensilla by means of

the "tip-recording" technique (Hodgson et al., 1955). The reference electrode, a thin Ag/AgCl, was inserted into the head and gently pushed into the maxillary-labial complex to fix the maxillae in a prognathous position. The recording electrode, a glass micropipette (tip diameter 20 μm), filled with the stimulating solution, was placed over the sensillum tip. All signals were recorded with a high input impedance (10^{15} Ω) electrometer (WPI, Duo 773), band-pass filtered (0.1 - 3 KHz), digitized by means of an Axon Digidata 1440A A/D acquisition system (sampling rate 10 KHz) and stored on PC for later analysis.

2.3 Data analysis

Recordings typically lasted 2-3 s, but spike analysis was performed in the interval 10 - 1010 ms after contact with the sensillum, the first 10 ms being skipped as containing the contact artifact. The 1st second of the discharges was chosen as representative of the phasic/phasic-tonic parts of the response (Dethier and Crnjar, 1982; Inoue et al., 2009) and the spikes sorting and counting were performed by means of the Clampfit 10.0 software, based on earlier studies (Dolzer et al., 2003; Dulcis and Levine, 2005; Pézier et al., 2007; Sollai et al., 2014). By measuring the peak-antipeak amplitude and the duration of action potentials we have previously shown that the each maxillary styloconic sensillum in the larvae of *P. hospiton* houses four GRNs: S, M1 and M2, L. Three of these are sensitive to bitters: two in the lateral ("lat-L" and "lat-M2") and one in the medial sensillum ("med-M2") (Sollai et al. 2014).

2.4 Behavioural experiments

The feeding assay protocol, described in Sollai et al. (2014), involves five steps. (a) The larva is placed in a "food-deprivation arena" consisting of an inverted Petri dish covered with a clear plastic cylinder (13 cm in diameter and 7 cm tall) where it is kept without food for 30 min in order to standardize its "hunger" state. (b) The larva is transferred to the "test-arena", identical to the previous one except for a piece of cork (1cm in diameter, 4-5 mm high) taped to the center of

the Petri dish. Just prior to each test session, a glass-fiber disk (Whatman GF/A, 4.25 cm in diameter; Sigma-Aldrich, Italy) was pinned to the cork, and then moistened with 200 µl of bitter compound + 200 µl of *myo*-inositol 100 mM. (c) The larva is positioned on the edge of the disk and the assay starts when the caterpillar taps the disk surface with its chemosensilla. (d) After a 2 min period, the larva is removed from the "test-arena" and is transferred to a plastic cup for 30 min, where it has ad libitum access to its host-plant. (e) Finally, the larva is returned to the "food-deprivation arena" for 30 min, to start a new testing cycle. Each larva was tested with the three concentrations of all stimuli. Behavioural experiments were initially performed on intact insects and later, on the same caterpillars, after bilateral ablation of the lateral sensilla, done according to de Boer and Hanson (1987). The larvae were put on ice for 20 min, their head was blocked with a rubber gasket, and their sensilla were cut at the base by means of iridectomy scissors. Immediately after the ablation, the larvae were placed on their host-plant for 24 h. All larvae survived ablation and were then used for the behavioural tests.

To evaluate feeding behaviour we measured two parameters: (a) the latency to start feeding, measured as the time elapsed between initial tasting of the glass-filter disk and initiating feeding and (b) the total amount of disk area eaten during the 2 min feeding assay. To evaluate the disk area eaten we calculated the differences between the dried weight of each disk moistened with 400 µl of a test stimulus before (control) and after a 2 min feeding assay.

2.5 Stimuli

For all electrophysiological and behavioural experiments, taste solutions were prepared immediately before testing and were presented at room temperature. The chemical stimuli were purchased from Sigma-Aldrich, (Italy). To determine if all 3 bitter-sensitive neurons are always activated, we stimulated the lateral and medial sensilla of the gustatory system with 4 bitter substances that belong to different chemical classes: nicotine and caffeine (alkaloids), salicin (β-glucoside) and quercitrin (flavonoid), each at 3 different concentrations: nicotine, caffeine,

salicin at 0.1, 1, 10 mM, and quercitrin at 0.01, 0.1, 1 mM, chosen on the basis of data in the literature (Dethier and Kuch, 1971; Schoonhoven and van Loon, 2002). Although a higher concentration of salt stimulates deterrent cells and induces aversive behaviour, 50 mM KCl was used to dissolve all compounds in order to optimize recording conditions and signal-to-noise ratio for a better spike identification (Bernays and Chapman, 2001; del Campo and Miles, 2003; Glendinning et al., 2006; Inoue et al., 2009; Jørgensen et al., 2006; Sollai et al., 2014; Zhang et al., 2013).

Stimuli were applied in a randomized sequence except for 50 mM KCl that was tested first (control solution). A 3-min interval was allowed between consecutive stimulations to minimize adaptation phenomena. At the end of each sequence, 50 mM KCl was tested again to assess any shift in responsiveness; whenever relevant spike frequency variations were found (wider than 50%), the experiment was discarded: this occurred in less than 10% of the experiments.

In order to avoid any drift in solution concentration due to evaporation, a clean, dry piece of filter paper was used to draw fluid from the tip of recording/stimulating electrode just before each recording. After each test, the mouthparts of the insect were rinsed with distilled water and blotted dry. Finally, we recorded only from sensilla of one maxilla for each larva (N=18-24) and no preparation was used in more than one experiment.

Behavioural trials were conducted first on intact insects and than, on the same insects from which both lateral sensilla had been removed (lat-ablated insects). Intact insects (N=12) were tested with nicotine, caffeine, salicin (0.1, 1, 10 mM), and quercitrin (0.01, 0.1, 1 mM); only nicotine and caffeine were instead tested in the experiments with lat-ablated insects (N=12). All stimuli were dissolved in bidistilled water and tested with the addition of inositol 100 mM, which is known to stimulate feeding in other insects (Schoonhoven and van Loon., 2002). A phagostimulant was added to all glass fiber disks because preliminary behavioural tests showed that feeding did not start when disks were presented to the larvae with the bitter compound only, as reported for other insects (Shields et al., 2006)

2.6 Statistical analysis

Repeated-measures ANOVA was used to analyze, separately for each taste stimulus: a) the effect of increasing concentrations of bitter taste stimuli (nicotine, caffeine, salicin and quercitrin) on the spike frequency in the first second of discharges of lat-L, lat-M2 and med-M2 GRNs; b) the effect of the bitter compounds on feeding latency and on amount of food eaten.

Two-way ANOVA was used to verify: a) the presence of a rate or ensemble code between two taste stimuli; we analyzed the total number of spikes generated by each bitter-sensitive GRN in the first second of stimulation. We inferred the presence of a rate code, e.g. between nicotine and caffeine, if there was a significant main effect of the taste stimulus on the spikes frequency and the presence of an ensmble code if there was a significant interaction of Stimulus × GRN; b) the presence of a temporal code; time-intensity (T-I) curves (i.e., the number of action potentials during each successive 100 ms of stimulation during the first second of activity) were obtained separately for each taste stimulus and bitter-sensitive GRN. We inferred the presence of a temporal code (e.g., between nicotine and caffeine), if there was a significant interaction of Time × Stimulus; c) the presence of a spatio-temporal code; time-intensity curves (T-I) of each GRN were considered separately for each stimulus, and we asked if the T-I curve produced by a GRN was different from that produced by the other GRNs. We inferred the presence of a spatio-temporal code (e.g., between nicotine and caffeine), if the curves T-I of a taste stimulus produced a significant interaction of Time × GRN, while those of another stimulus produced a non-significant interaction.

Data were checked for the assumptions of homogeneity of variance, normality and sphericity (when applicable). When the sphericity assumption was violated, a Greenhouse-Geisser correction or Huynh-Feldt correction was applied in order to modify the degrees of freedom. Post-hoc comparisons were conducted with the Tukey test, unless the assumption of homogeneity of variance was violated, in which case the Duncan's test was used. Statistical analyses were made using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK,

USA). *P* values < 0.05 were considered significant.

2.7 Permits

Required permits were obtained for *Papilio hospiton.* Specimens were collected in Sardinia in the spring of 2012, in compliance with the permit issued on 28 May 2012 (Ref. # 0010888) to Roberto Crnjar and his collaborators, by "Ministero dell'Ambiente e della Protezione del Territorio e del Mare" (Italian Board of Environment and Protection of Land and Sea), in derogation from the provisions set out in the regulation DPR 357/97 concerning the application of the "Council Directive 92/43/EEC of 21 May 1992 on conservation of natural habitats and of wild fauna and flora". No specific permits were required for *Ferula communis,* as it is not endangered or protected specie.

3. Results

3.1 Spikes activity of the bitter-sensitive GRNs and dose-response relationship.

Samples of spike discharges of the activity of bitter-sensitive GRNs, recorded from the lateral and medial styloconic sensilla, in response to test stimuli are shown in figures 1 and 2.

To test for a dose-response relationship, we analyzed the spike activity evoked in the first second of the discharge for each bitter-sensitive GRN (lat-L, lat-M2 and med-M2) to increasing concentrations of nicotine, caffeine, salicin and quercitrin, by using a repeated-measures ANOVA (Fig. 3).

For lat-L GRN, repeated-measures ANOVA showed a significant effect of concentration on the spike frequency in response to nicotine ($F_{[1.7,44.4]}$=35.992; p<0.000001) and caffeine ($F_{[2,54]}$=7.094; p=0.00184). On the contrary, no spike activity was found after stimulation with salicin and quercitrin. These findings, together with the analysis of spike traces (Figs. 1, 2), indicate that the lat-L GRN was activated by nicotine and caffeine, but not by salicin and quercitrin.

For lat-M2 GRN, repeated-measures ANOVA showed a significant effect of concentration on the spike frequency in response to all bitter compounds tested (nicotine $F_{[2,50]}$=4.3602; p=0.017, caffeine $F_{[2,50]}$=12.277; p=0.00005, salicin $F_{[2,46]}$=19.714; p<0.000001, quercitrin $F_{[1.66,41.5]}$=4.169; p=0.028). Finally, also for med-M2 GRN, repeated-measures ANOVA revealed a significant effect of concentration on the spike frequency in response to all bitter compounds tested (nicotine $F_{[1.6,39.6]}$=13.233; p=0.00013, caffeine $F_{[2,50]}$=16.316; p<0.000001, salicin $F_{[2,42]}$=7.689; p=0.00143, quercitrin $F_{[2,46]}$=6.975; p=0.0023). These findings, together with the analysis of spike traces (Figs. 1, 2), indicate that nicotine, caffeine, salicin and quercitrin activate both lat-M2 and med-M2 GRNs.

3.2 Sensory codes mediating the bitter taste discrimination

We asked if the bitter-sensitive neurons could discriminate between different bitter compounds by means of a rate, ensemble, temporal and/or spatio-temporal code. We restricted the analysis to the highest concentration used, and we made the comparison between pairs of stimuli on the basis of the number of bitter-sensitive neurons activated: caffeine/nicotine and salicin/quercitrin. To verify the presence of a different rate or ensemble code, we analyzed the total number of spikes evoked in the first second of stimulation with each of the four bitter compounds tested (Fig. 4). In the caffeine/nicotine comparison, there was a significant main effect of taste stimulus ($F_{[1,141]}$=16.668; p=0.00007) and a significant interaction of Stimulus × GRN on the spike frequency ($F_{[2,139]}$=16.636; p=0.00001). These results indicate that caffeine and nicotine generated both a different rate (i.e., nicotine > caffeine) and ensemble codes. On the contrary, in the salicin/quercitrin comparison, we found neither a main effect of taste stimulus ($F_{[1,76]}$=0.0473; p=0.8284) nor a significant interaction of Stimulus × GRN on the spike frequency ($F_{[1,75]}$=0.0685; p=0.7943). These results indicate that salicin and quercitrin did not generate a different rate or ensemble code.

To verify the presence of a different temporal code, we analyzed the T-I curves for each bitter

stimulus and evaluated the presence of a significant interaction of Stimulus × Time, by using two-way ANOVA. A significant interaction of Stimulus × Time was found in the caffeine/nicotine comparison ($F_{[9,1390]}$=2.6494; p=0.0048), but not in the salicin/quercitrin one ($F_{[9,790]}$=1.5537; p=0.1251) (Fig. 5A). These results indicate that caffeine and nicotine, but not salicin and quercitrin, generated different temporal codes. Finally, to verify the presence of a different spatio-temporal code, we analyzed the T-I curves produced by each GRN sensitive to bitter, separately for each taste stimulus (Fig. 5B). For nicotine, there was a significant interaction of Time × GRN ($F_{[18,640]}$=2.9119; p=0.00005): this result shows that nicotine evoked non-parallel T-I curves in the three bitter-sensitive GRNs. Instead, for caffeine ($F_{[18,750]}$=0.4861; p=0.9644), salicin ($F_{[9,410]}$=1.5402; p=0.1315)and quercitrin ($F_{[9,380]}$=0.5562; p=0.8327), the interaction of Time × GRN was non-significant. These results show that caffeine, salicin and quercitrin evoked, each, T-I curves in the bitter-sensitive GRNs that were essentially parallel to one other. These findings indicate that nicotine generated a different spatio-temporal code with respect to caffeine, salicin and quercitrin.

3.3 Effect of bitter compounds on feeding behaviour

For intact insects, mean values ± s.e.m. of time required by larvae to start eating (feeding latency) disks moistened with nicotine, caffeine, salicin (0.1, 1 and 10 mM) and quercitrin (0.01, 0.1 and 1 mM), and of percentage of remaining dried weight of disks (RDW) after a 2-min feeding trial as compared to pre-trial values (control, 100% in the graphs), are shown in figure 6. Results indicate that the larvae fed on the disks wetted with all concentrations of salicin and quercitrin, but rejected those containing nicotine and caffeine. Repeated-measures ANOVA revealed a significant increase of feeding latency ($F_{[1.1,11.9]}$=5.9402; p=0.0029) and of RDW percentage ($F_{[2,22]}$=4.0178; p=0.033), with increasing concentrations of salicin; also, repeated-measures ANOVA showed a significant increase of RDW percentage ($F_{[2,22]}$=4.9969; p=0.0162), with increasing concentrations of quercitrin, while no difference was found on the feeding

295 latency.

296 For lat-ablated insects, figure 6 shows the mean values ± s.e.m. of the feeding latency on the
297 disks moistened with nicotine and caffeine (0.1, 1 and 10 mM), and of RDW percentage after a
298 2-min feeding trial as compared to pre-trial values (control, 100% in the graphs). Repeated-
299 measures ANOVA revealed a significant increase of feeding latency (nicotine $F_{[1.1,12.3]}$=24.637;
300 p=0.00023, caffeine $F_{[1.4,15.3]}$=3.7414; p=0.04) and of RDW percentage (nicotine $F_{[2,22]}$=14.934;
301 p=0.00008, caffeine $F_{[2,22]}$=4,33; p=0.026), with increasing concentrations of nicotine and
302 caffeine.

303 These results suggest that the lateral sensillum, and more probably the bitter-sensitive lat-L
304 GRN, plays a role in the discriminating capability of different bitter compounds.

305

306 **4. Discussion**

307 The main role of the bitter sensitive GRNs is to provide information about the presence of
308 potentially harmful compounds. In the larval styloconic sensilla of *P. hospiton*, we have
309 previously identified 3 bitter-sensitive GRNs: two in the lateral (lat-L, lat-M2) and one in the
310 medial (med-M2). To better assess the sensitivity profiles of these 3 bitter-sensitive neurons, we
311 stimulated the lateral and medial maxillary styloconic sensilla of both species with 4 bitter
312 substances belonging to different chemical classes: nicotine and caffeine (two alkaloids), salicin
313 (a β-glucoside) and quercitrin (a flavonoid). The dose-response relationships that we found show
314 that salicin and quercitrin activate only two bitter-sensitive GRNs (lat-M2 and med-M2), while
315 nicotine and caffeine also the third one (lat-L). This result suggests that a spatial coding
316 mechanism (two GRNs activated by salicin and quercitrin and three by nicotine and caffeine) is
317 used by *P. hospiton* larvae to distinguish between different bitter compounds. Since it is known
318 that alkaloids are toxic compounds, while salicin and quercitrin are unpalatable but non-toxic
319 compound (Després et al., 2007; Detzel and Wink, 1993; Pentzold et al., 2014; Schuler, 1996;
320 Steppuhn et al., 2004), we speculate that the activation of lat-M2 and med-M2 GRNs may signal

the presence of non-noxious bitter compounds, and that lat-L GRN (activated only by nicotine and caffeine) may act as a “labeled-line” which indicates the presence of toxic compounds.

Similar results have been reported in *D. melanogaster*, where some bitter taste stimuli evoke responses from different subsets of neurons (Weiss et al., 2011) and in *P. brassicae*, where one neuron is very sensitive and specialized towards cardenolids and unresponsive to all other bitter compounds that excite, instead, the other neuron (van Loon and Schoonhoven, 1999). In other insects, however, all bitter compounds tested activate the same number of neurons and the process of taste discrimination is mediated by different temporal codes (Glendinning et al., 2006). On this basis, we asked whether other paradigms of neural coding, such as rate, ensemble, temporal and spatio-temporal codes, could help in the process of discrimination between those pairs of stimuli that activated the same number of bitter-sensitive GRNs. The results show that the larvae are able to discriminate between nicotine and caffeine by means of different coding paradigms: firing pattern, rate and ensemble. Instead, no coding paradigm was found to provide a basis for neural discrimination between salicin and quercitrin. These findings suggest *P. hospiton* is able to discriminate also between toxic bitter compounds, but not between non-toxic ones, by means of different coding paradigms.

Besides, we found that nicotine is discriminated from any of the other bitter stimuli also by means of a spatio-temporal code. In fact, the analysis showed that the T-I curves generated by each bitter-sensitive GRNs in response to nicotine are not parallel, with lat-L and med-M2 neurons showing a phasic-tonic trend, while lat-M2 neuron basically shows a tonic pattern of discharge. Conversely, the T-I curves obtained in response to the other stimuli, are phasic-tonic and parallel. Insects endowed with a small number of bitter sensitive GRNs, such as *P. hospiton*, should benefit from a choice of additional coding paradigms in taste discrimination. The availability of other coding mechanisms, complementing spatial coding, would potentially allow discrimination of a wider variety of bitter compounds, as suggested in other insects (Dethier and Crnjar, 1982; Glendinning et al., 2006; Marella et al., 2006; Weiss et al., 2011).

Previous studies have shown that differences in the sensitivity to bitter substances evoke different behavioural responses. In *P. brassicae*, for example, the information from the specialist deterrent cell leads the caterpillars to reject the food source, while the generalist deterrent cell does not always cause a rejection (van Loon and Schoonhoven, 1999). Our behavioural data indicate that larvae with the complete set of chemosensilla only eat discs moistened with salicin and quercitrin, at all concentrations tested, while rejecting altogether those containing caffeine and nicotine. The aversive response to alkaloids was completely abolished upon ablation of the bilateral pairs of the lateral sensilla, supporting the hypothesis that the information rising from the lat-L GRN plays a role in the taste discriminating process. However, while the larvae ate disks moistened with lower concentrations of caffeine and nicotine, they just took a few bites on those with the same compounds at the highest concentration tested, as shown by the fact that the percentage of disk remaining after a 2-min feeding time was not statistically different from the control. This result suggests that other mechanisms are involved in controlling the feeding once it has begun. It has been proposed that herbivorous insects possess both pre-ingestive (mediated by peripheral taste system) and rapidly acting post-ingestive mechanisms; the latter include feedback mechanisms at the level of the central nervous system, such as the inhibition of chewing and/or of the center of feeding control. The post-ingestive mechanisms should help the insect regulate the intake of toxic compounds at physiologically tolerable levels, while it is producing the detoxifying enzyme, preventing it to ingest lethal doses of toxic compounds (Glendinning, 2002).

In conclusion, these results suggest that the larvae of *P. hospiton* may be able to discriminate among compounds belonging to the bitter taste modality, but that the neural coding mechanism used may be more complex than a simple system contemplating only a labeled-line and/or an across neuron pattern model. In detail, they seem to use a spatial coding mechanism (labeled-line code) for discriminating between bitter toxic and non-toxic compounds; in fact, the lat-L neuron seems to detect specifically the presence and mediate the rejection behaviour of toxic

compounds. In addition, toxic compounds, but not non-toxic ones, can be further discriminated from each other by means of other paradigms of neural coding, such as frequency, ensemble, temporal and spatio-temporal codes. Thus, the process of discrimination between compounds that activate the same bitter-sensitive taste neurons, as already shown in *M. sexta* and *Bombyx mori* (Asaoka, 2000; Glendinning et al., 2006), appears to be a widespread taxonomically phenomenon.

As regards functional implications, the discrimination ability between different bitter compounds is particularly useful for herbivorous animals, because plants produce and accumulate in their nutrients tissues (such as leaves, roots, etc.) a large variety of secondary metabolites that humans taste bitter, evoking in many species of animals, a behavioural response of aversion to food. This ability could avoid to elicit false alarms (i.e., causes an insect to reject a relatively harmless food) and be very useful in habitats where plants contain poisonous or while exploring new non-family environments. This would make the larvae capable of selecting, as possible food substrates, plants containing harmless compounds (Glendinning et al., 2006; Weiss et al., 2011).

Acknowledgements

We are grateful to Dr. Marco Melis, Dept of Biomedical Sciences, University of Cagliari, for technical assistance. This work was supported by the Regione Autonoma della Sardegna [CRP-59859] and the Fondazione Banco di Sardegna [2012/0245].

Conflict of interest

There are no financial and personal relationships with other people or organizations that may lead to a conflict of interest.

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Legends of Figures

Fig. 1 – Sample traces showing spike firing frequency of a lateral styloconic sensillum following stimulation with nicotine, caffeine, salicin (10 mM) and quercitrin (1 mM). Top traces are time scale expansions of section within hatched lines in bottom trace.

Fig. 2 – Sample traces showing spike firing frequency of a medial styloconic sensillum in following stimulation with nicotine, caffeine, salicin (10 mM) and quercitrin (1 mM). Top traces are time scale expansions of section within hatched lines in bottom trace.

Fig. 3 – Mean values ± s.e.m. of spike activity evoked in bitter-sensitive GRNs following stimulation with increasing concentrations with nicotine, caffeine, salicin (0.1, 1, 10 mM) and quercitrin (0.01, 0.1, 1 mM). N=18-24. Circles indicate the response to 50 mM KCl (K). Symbols indicate significant differences between a concentration and the next lower (* = $p<0.05$; Tukey test subsequent to repeated-measures ANOVA; # = $p<0.01$; Duncan's test subsequent to repeated-measures ANOVA).

Fig. 4 – Mean values ± s.e.m. of total number of spikes evoked (A) in all three bitter-sensitive GRNs and (B) in each bitter-sensitive GRN during the first second of stimulation with nicotine, caffeine, salicin (10 mM) and quercitrin (1 mM). N=18-24.
(A) # = significant differences between the pairs of taste stimuli considered ($p<0.0001$; Duncan's test subsequent to main effect ANOVA); (B) # = significant differences between the spike activity of same GRN in response to different taste stimuli considered in pairs ($p<0.00001$; Duncan's test subsequent to two-way ANOVA).

Fig. 5 – (A) Time-Intensity curves (i.e. number of spikes during 10 consecutive 100 ms intervals) elicited by nicotine, caffeine, salicin (10 mM) and quercitrin (1 mM). N=18-24.

(#) significant differences of the number of spikes between corresponding 100 ms ($p<0.05$; Duncan's test subsequent to two-way ANOVA).

(B) Time-Intensity curves from each bitter-sensitive GRNs elicited by nicotine, caffeine, salicin (10 mM) and quercitrin (1 mM) in both species. N=18-24.

Fig. 6 – (A) Mean values ± s.e.m. of time needed to caterpillars to start eating (feeding latency) glass-filter disks moistened with nicotine, caffeine, salicin (0.1, 1, 10 mM) and quercitrin (0.01, 0.1, 1 mM) dissolved in water, in both intact and lat-ablated insects. N=12 for both intact and lat-ablated insects. Symbols indicate statistical differences between a concentration and the next lower (* = $p<0.05$; Tukey test subsequent to repeated-measures ANOVA; # = $p<0.005$; Duncan's test subsequent to repeated-measures ANOVA). (**§**) never started to feed.

(B) Mean values ± s.e.m. of percentage of remaining weight of disks moistened with nicotine, caffeine, salicin (0.1, 1, 10 mM) and quercitrin (0.01, 0.1, 1 mM) dissolved in water, in both intact and lat-ablated insects, after the 2-min feeding trial as compared to pre-trial values (control, 100%). N=12 for both intact and lat-ablated insects. Symbols indicate statistical differences from the control (* = $p<0.01$; Tukey test subsequent to repeated-measures ANOVA; # = $p<0.005$; Duncan's test subsequent to repeated-measures ANOVA).

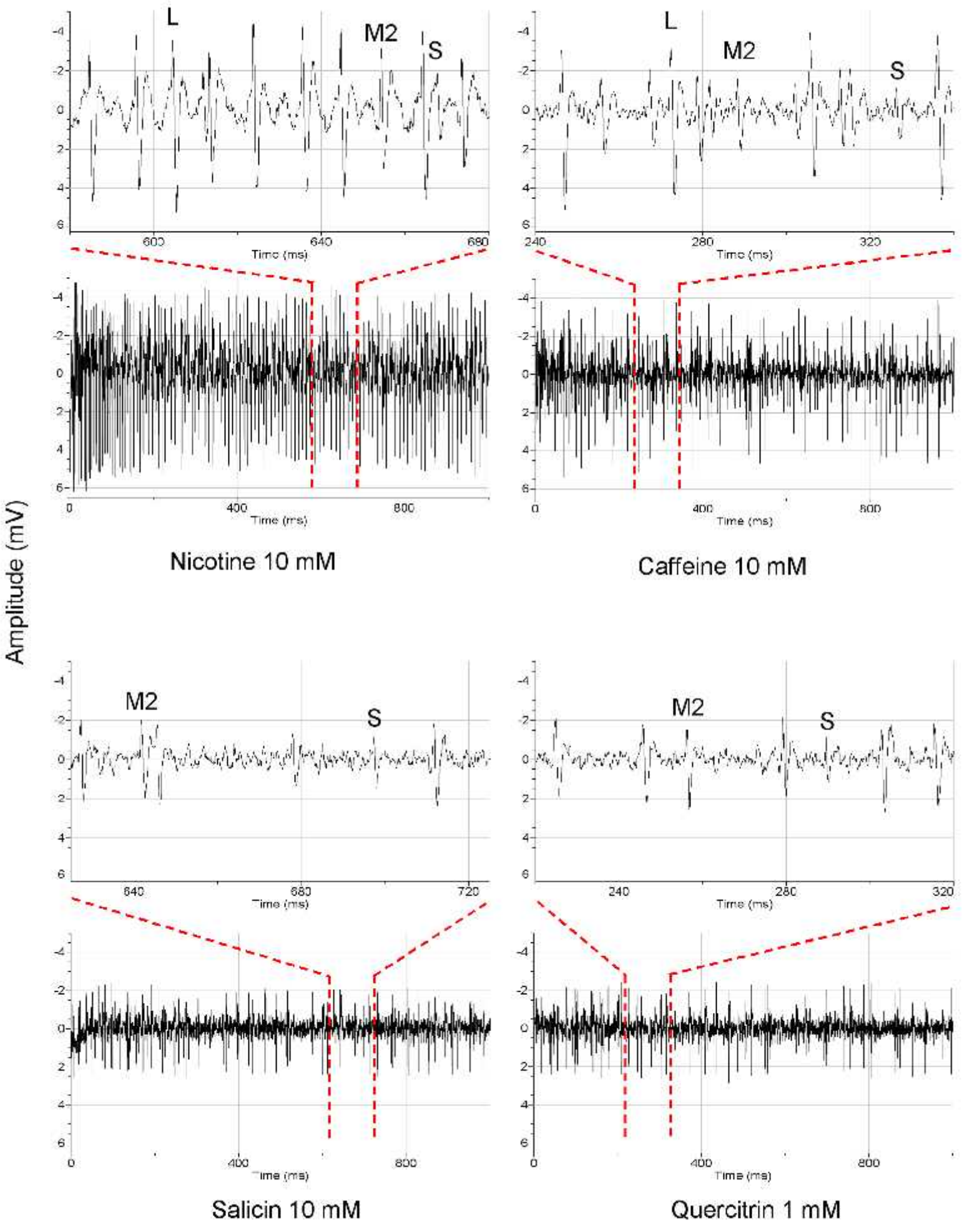

Lateral sensillum
L
M2
S
Time (ms)
Amplitude (mV)
Nicotine 10 mM
Caffeine 10 mM
Salicin 10 mM
Quercitrin 1 mM

Figure 1

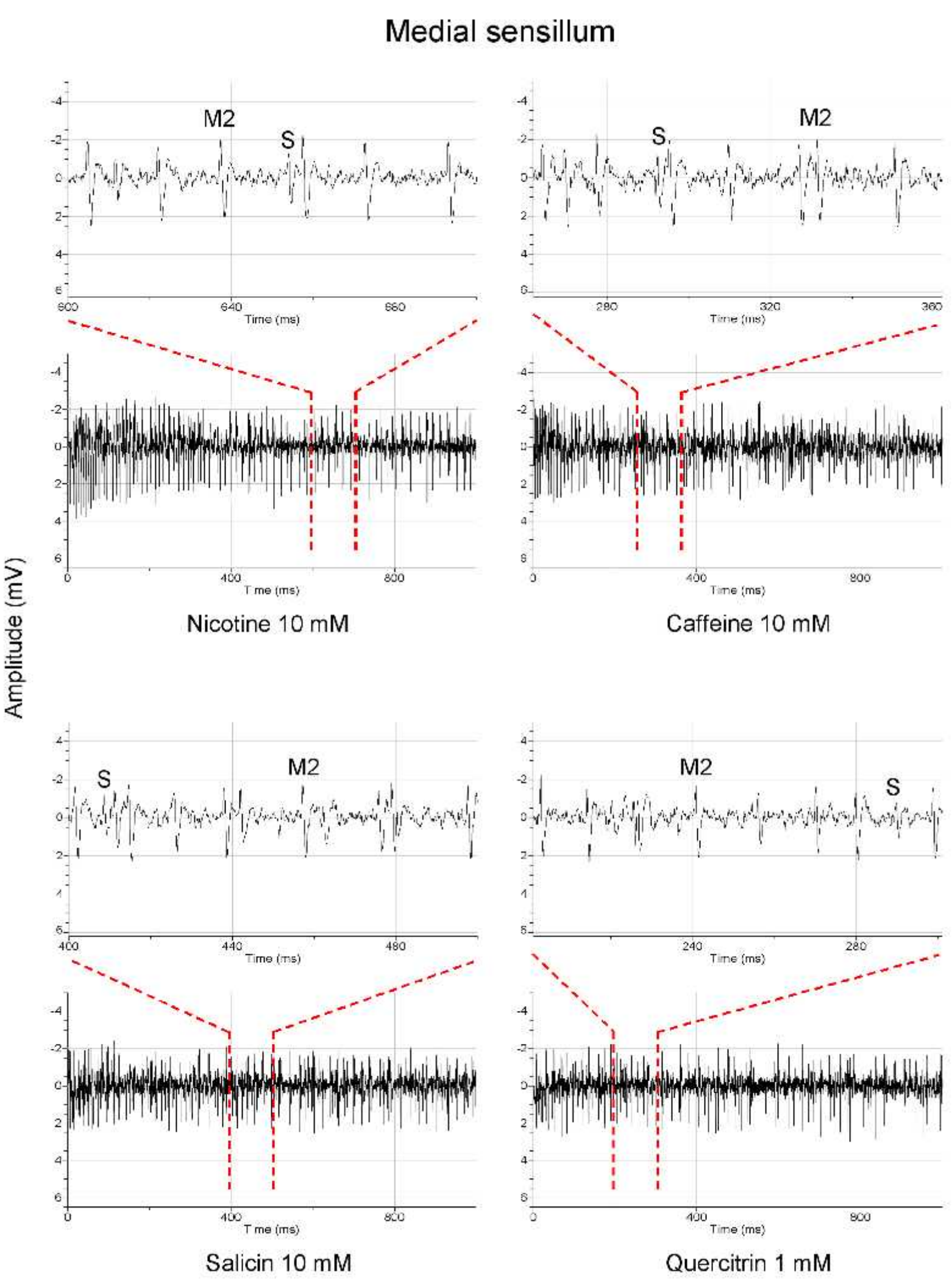

Medial sensillum
M2
S
Time (ms)
Nicotine 10 mM
Caffeine 10 mM
Salicin 10 mM
Quercitrin 1 mM
Amplitude (mV)

Figure 2

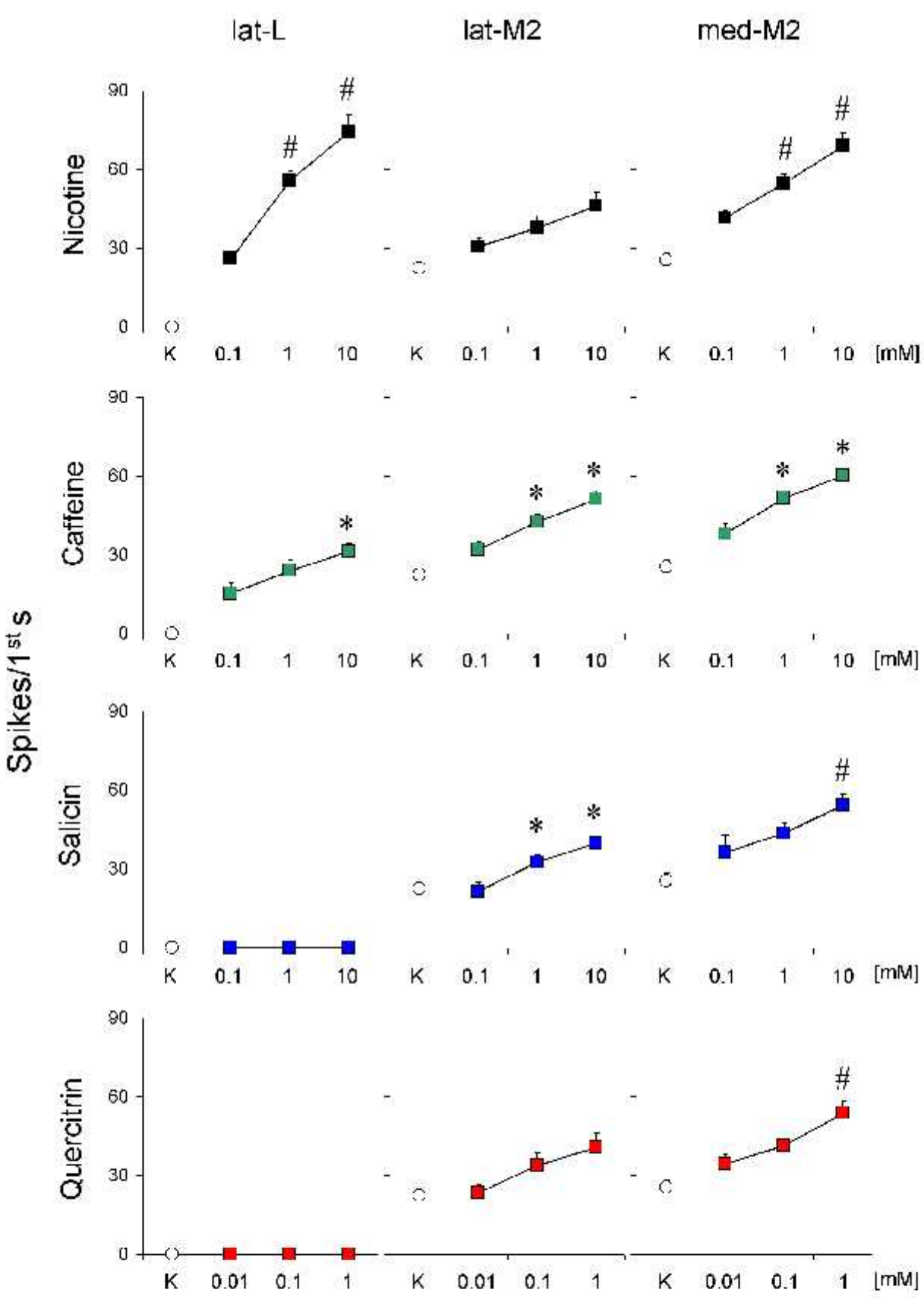
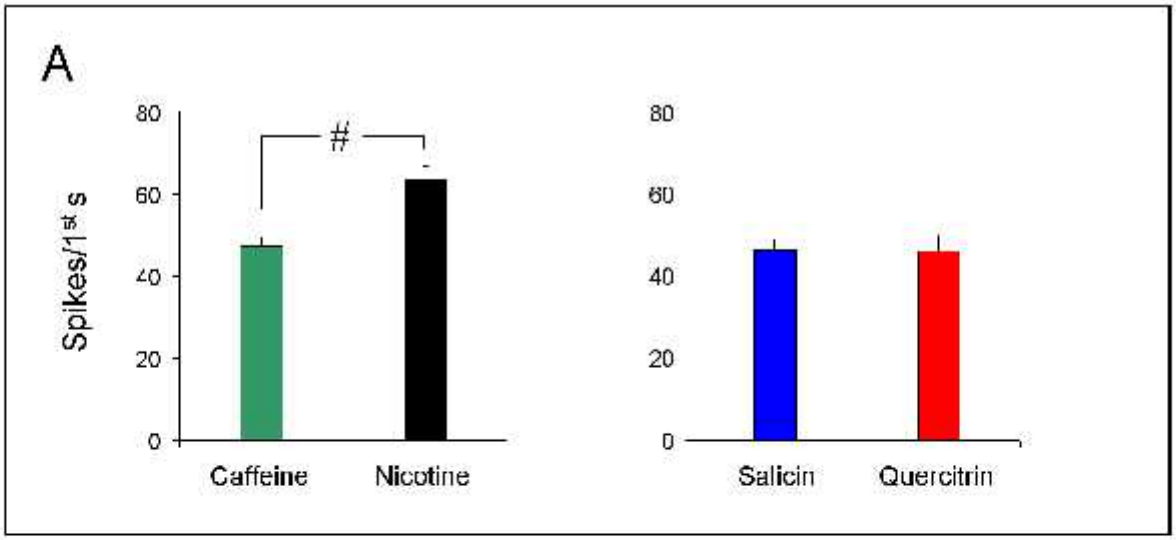

lat-L
lat-M2
med-M2
Nicotine
Caffeine
Salicin
Quercitrin
Spikes/1st s
90
60
30
0
K
0.1
1
10
0.01
[mM]
#
*

Figure 3


A
Spikes/1st s
80
60
40
20
0
#
Caffeine
Nicotine
Salicin
Quercitrin

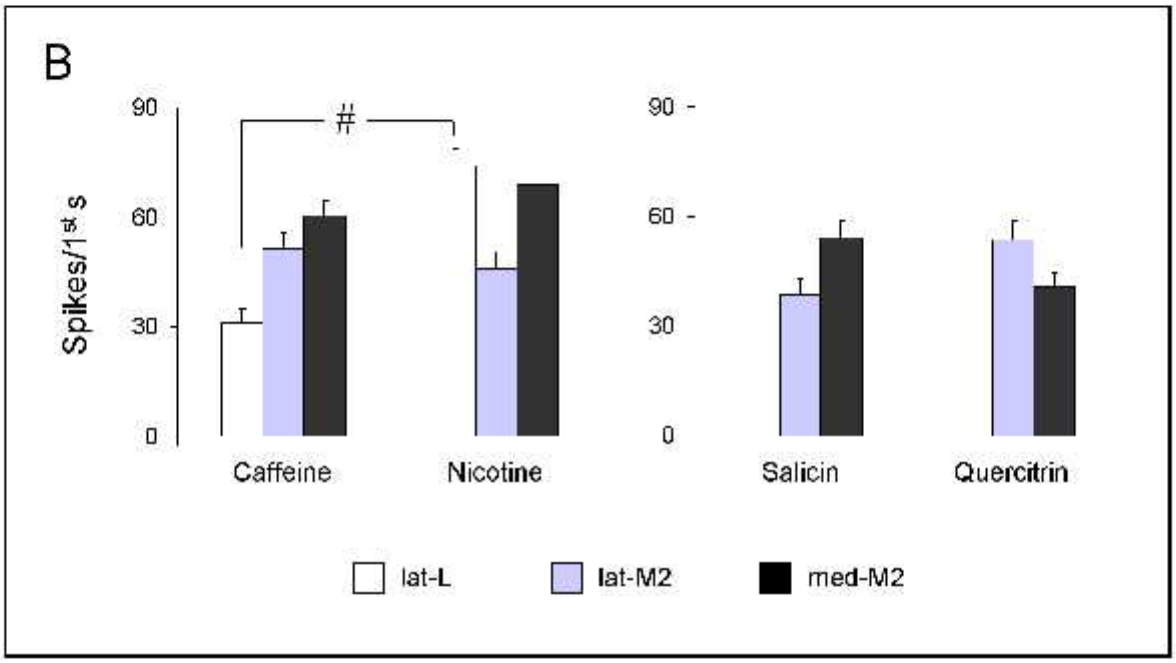
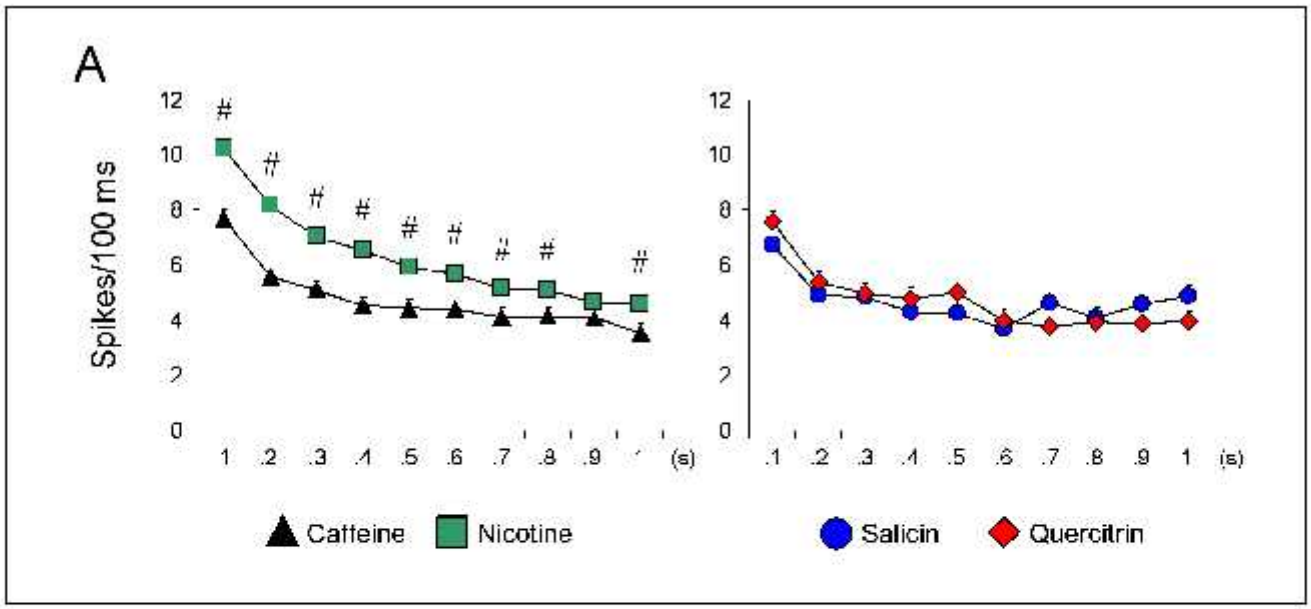

B
Spikes/1st s
90
60
30
0
#
Caffeine
Nicotine
Salicin
Quercitrin
lat-L
lat-M2
med-M2

Figure 6


A
Spikes/100 ms
12
10
8
6
4
2
0
#
1 .2 .3 .4 .5 .6 .7 .8 .9 (s)
Caffeine
Nicotine
Salicin
Quercitrin

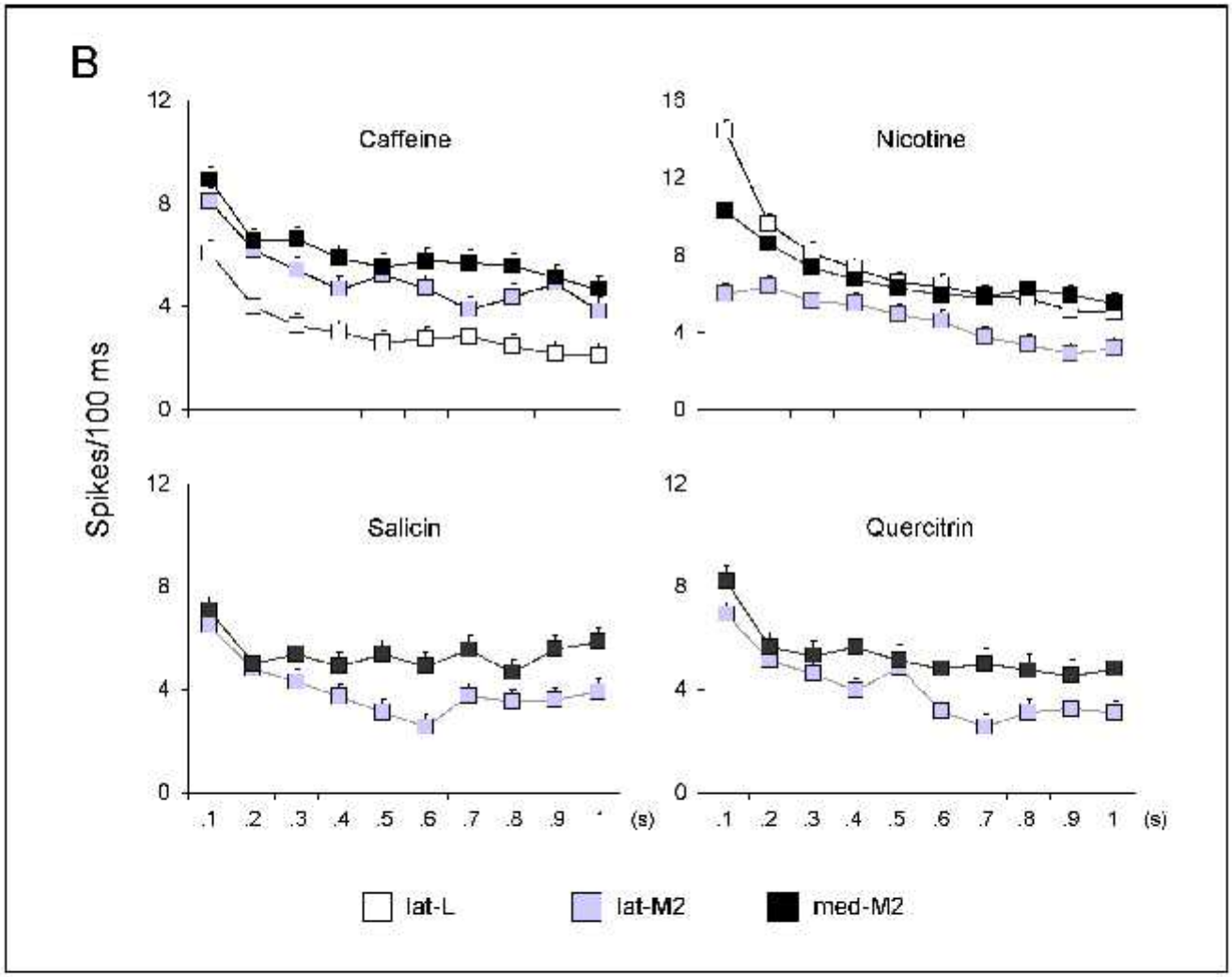

B
Spikes/100 ms
Caffeine
Nicotine
Salicin
Quercitrin
12
8
4
0
.1 .2 .3 .4 .5 .6 .7 .8 .9 1 (s)
lat-L
lat-M2
med-M2

Figure 5

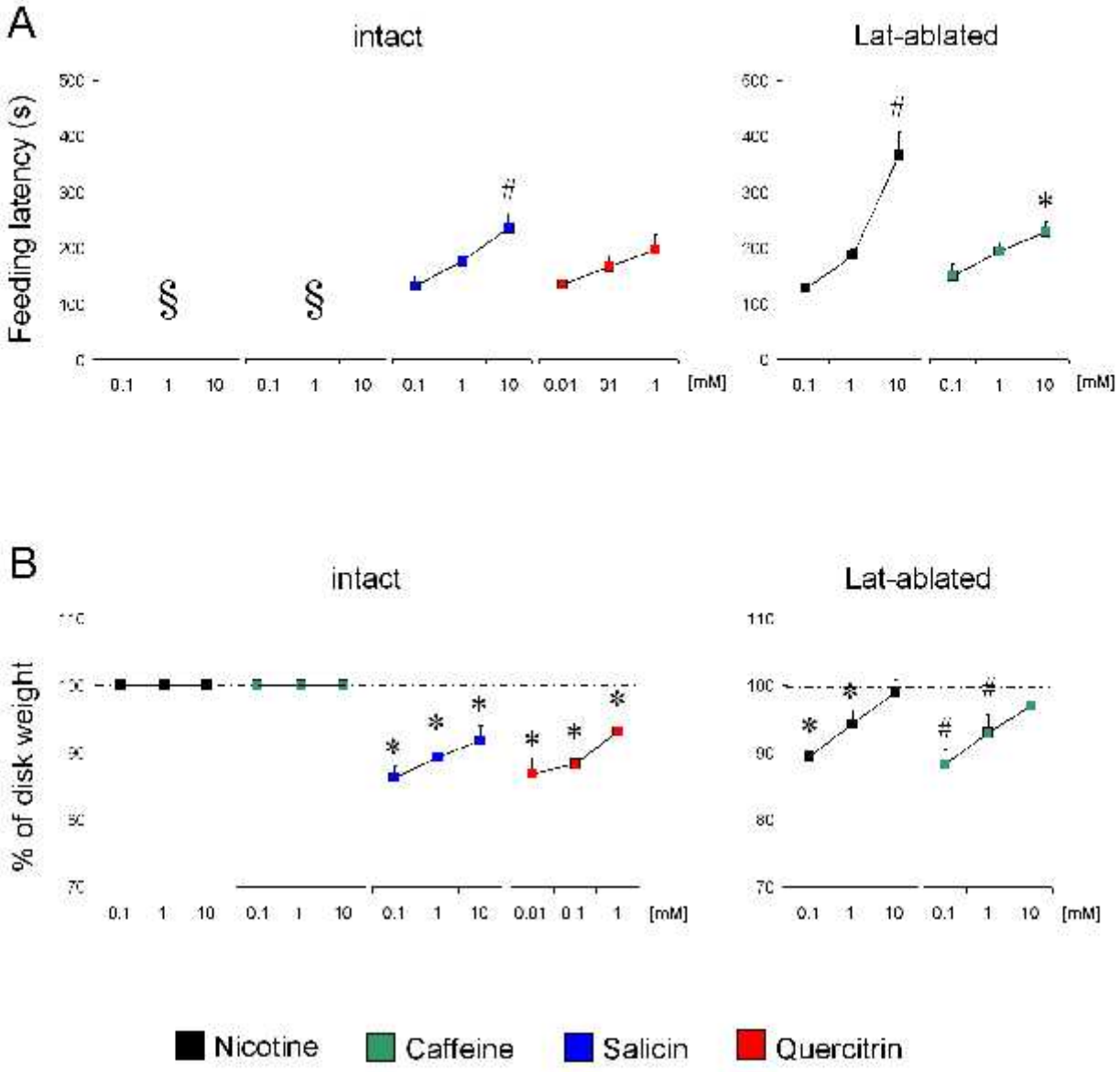

A
intact
Lat-ablated
Feeding latency (s)
500
400
300
200
100
0
§
§
#
#
*
0.1
1
10
0.01
[mM]
B
intact
Lat-ablated
% of disk weight
110
100
90
80
70
*
#
Nicotine
Caffeine
Salicin
Quercitrin

Figure 6