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VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS

--Manuscript Draft--

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Abstract:	Background. Dopamine is reduced in the brain of rats treated with fipronil, a broad-spectrum insecticide. VGF (no acronym) is a neurotrophin-inducible protein expressed as the 75kDa form (precursor or pro-VGF) or its truncated peptides. VGF immunostaining has been revealed using an antibody against the C-terminal nonapeptide of the rat pro-VGF in the nerve terminals of the rat substantia nigra, where it was reduced after 6-hydroxydopamine treatment. It is unknown whether pro-VGF and/or its shortened peptides are present in these neurons. Therefore, the aim of this study was first to determine which types of VGF are expressed in the normal substantia nigra (and striatum) and then to determine VGF modulations and whether they occur in parallel with locomotor changes after fipronil injection. Methods. Rats were divided into two groups that received a unilateral intranigral infusion of either vehicle (i.e., dimethyl sulfoxide, DMSO) or fipronil (25 µg), and then were tested for locomotor activity. An untreated group of rats (n=4) was used for identification of the VGF fragments using high performance liquid chromatography-mass spectrometry and western blot, while changes in treated groups (fipronil vs DMSO, each n=6) were investigated by immunohistochemistry using an antibody against the rat pro-VGF C-terminal nonapeptide in parallel with the anti-tyrosine hydroxylase antibody. Results. In untreated rats, the VGF C-terminal antibody identified mostly a 75kDa band in the substantia nigra and striatum, supporting the finding of high-resolution mass spectrometry, which revealed fragments covering the majority of the pro-VGF sequence. Furthermore, several shortened VGF C-terminal forms (varying from 10 to 55 kDa) were also found by western blot, while high-resolution mass spectrometry revealed a C-terminal peptide overlapping the immunogen used to create the VGF antibody in both the substantia nigra and the striatum. In the substantia nigra of fipronil-treated rats, immunostaining for tyrosine hydroxylase and VGF was reduced compared to DMSO rat group, and this was related with significant changes in locomotor activity. Fipronil has the ability to modulate the production of pro-VGF and/or its C-terminal truncated peptides in the nigrostriatal system indicating its intimate interaction with the dopaminergic mechanisms and implying a potential function in modulating locomotor activity.

Suggested Reviewers:	Manuel Friese, MD Professor, University of Humburg inims@zmnh.uni-hamburg.de expert in neurodegeneration
	Koen Poesen, MD Professor, Catholic University of Leuven Faculty of Medicine koen.poesen@uzleuven.be expert in biomarkers

COVER LETTER

Dear Editor/s

Please find attached the revised manuscript titled:

VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS by the authors:

Elias Manca, Barbara Noli, Giulia Corda, Majda El-Hassani, Antonio Manai, Fabrizio Sanna, Antonio Argiolas, Maria Rosaria Melis, Barbara Manconi, Cristina Contini, and Cristina Cocco

The paper looking at the alterations of VGF proteins in rats treated with fipronil has been extensively changed in response to the referee's point-by-point requests. We hope that this version is greatly improved and ready for publishing in Annals of Anatomy in its current state.

Yours Faithfully

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Ms. Ref. No.: AANAT5497
Title: VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS
Annals of Anatomy

Dear Dr. Cocco,

The reviewers have commented on your above paper. They indicated that it is not acceptable for publication in its present form.

However, if you feel that you can suitably address the reviewers' comments , I invite you to revise and resubmit your manuscript.

Please carefully address the issues raised in the comments.

If you are submitting a revised manuscript, please also:

a) outline each change made (point by point) as raised in the reviewer comments

AND/OR

b) provide a suitable rebuttal to each reviewer comment not addressed

To submit your revision, please do the following:

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Yours sincerely,

Friedrich Paulsen
Editor-in-Chief
Annals of Anatomy

Note: While submitting the revised manuscript, please double check the author names provided in the submission so that authorship related changes are made in the revision stage. If your manuscript is accepted, any authorship change will involve approval from co-authors and respective editor handling the submission and this may cause a significant delay in publishing your manuscript.

Reviewers' comments:

Reviewer #1: In the manuscript entitled VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS the authors investigated the consequences of unilateral intranigral fipronil on several molecular parameters in various brain regions (CPu, SN, hypothalamus, nucleus accumbens) and motor behavior. The aim of this study was (i) to determine which types of VGF are expressed in the normal substantia nigra (and striatum), and (ii) to determine various VGF modulations (including pro-VGF and/or its truncated forms) and (iii) if they occur in parallel with locomotor changes after fipronil. The main focus of the manuscript is on molecular science of the VGFs. The molecular findings are partly new. Main results are: Fipronil induced a decrease in immunostaining for both TH and VGF in the substantia nigra (but not in the striatum), in parallel with significant changes in spontaneous locomotor activity. It is concluded, that fipronil is able to affect the release of pro-VGF and/or its C-terminal truncated peptides, confirming its close association with the dopaminergic system and suggesting a possible role in altering locomotor activity.

All in all, rather few animals (n=16) divided into 3 groups (completely untreated, DMSO unilateral intranigral, unilateral intranigral fipronil were used. The molecular results are interesting, the statistic of the very few samples (for me) are a bit questionable.

Answer: We apologize for not making this point obvious in the paper. In total, 28 rats were used for the study. Four of them were untreated, i.e., they did not undergo any surgical (or other) treatments and were only utilized for VGF peptide characterization (WB and proteomic), while the other 24 were stereotaxically injected into the substantia nigra with DMSO or fipronil (n=12 each group) and used for the locomotor activity tests (see on this point the degrees of freedom of the ANOVA that were previously reported in the paragraph on the locomotor activity results of the first paper version). Of them, six rats from each of the two treatment groups (i.e., six DSMO-treated and six fipronil-treated rats) were selected and effectively used for the IHC assays for the determination of TH and VGF immunostaining. The remaining rats were not chosen because they were either clearly not well perfused (brain still maintained red vassels, therefore IHC was not performed) or were only partially perfused, making it difficult to detect any positive labelling when doing IHC.
This point has been made clearer in the Material and Methods section of the revised manuscript and throughout the MS whenever appropriate.
As regards the number of rats used, we would point out that in the present study our primary aim was to investigate the potential involvement of VGF peptides in the intranigral lesion induced by fipronil. To this aim, we aimed at replicating the lesion model that was already extensively investigated by our group in previous work (Bharatiya et al., 2020; doi: 10.1016/j.bbr.2020.112562), ensuring that the animals used here were comparable to that of our previous study in terms of motor alterations and TH expression in the SN.

Hence, based on these considerations, and according to the 3Rs principles, we aim to minimize the number of animals used. To this scope, sample size calculations were performed to ensure adequate experimental group numbers to be used in the study. Accordingly, we found that a number of 6 rats/group would be sufficient to detect significant differences between groups in the IHC assays (t test, effect size d = 2, power (1-β) = 0.80, α = 0.05), while a number of 12 rats would be sufficient to detect significant differences between groups in the locomotor activity tests (t test, effect size d = 1.2, power (1-β) = 0.80, α = 0.05). These calculations have been based on prior studies using similar protocols (for instance, Angioni et al., 2016; doi: 10.1016/j.yhbeh.2016.05.012; Sanna et al., 2021; doi: 10.1016/j.brainres.2021.147705; Bharatiya et al., 2020a; doi: 10.1016/j.bbr.2020.112562) and were carried out by using the software G*Power 3.1 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologieund-arbeitspsychologie/gpower).
This information has been added in the Statistics section of the Material and Methods of the revised MS.

General:

always say how many animals were used for each result.

answer: The referee is correct; we have improved the animal number information throughout the new version of the manuscript.

Please use correct anatomical terms (The rat has no caudate: it has a CPu - just look at the atlas from Paxinos)

answer: the referee is correct, we have better described it

You partly speak of dopamine, but did not investigate it after fipronil or DMSO?

Answer: we did not do this in the present work, but it was extensively done in our previous work (Bharatiya et al., 2020a; doi: 10.1016/j.bbr.2020.112562) where we observed a significant decrease of dopamine tissue concentration in the striatum owing to fipronil injection at comparable doses and also in the SN and other brain areas after systemic intragastric chronic administration (Bharatiya et al., 2020b; doi: 10.3390/ijms21165711).
Here TH immunostaining was investigated as an indirect index of dopamine function in the nigrostriatal pathway (both in the SN and CPu).

You said: staining was found in a large number of SN neuron terminals containing both glutamic acid decarboxylase (GAD) and substance P- My question: are these terminals in the SN or in the projection areas of SN neurons (for example CPu)?

Answer: in a previously published article (Cocco et al, 2020), we documented, using triple staining, the existence in the substantia nigra of neuron terminals expressing Substance P, GAD, and VGF. Therefore, in the present study we aimed to reveal CPu cell bodies containing the three (VGF, Substance P, and GAD) in order to identify the projection areas of SN neurons within the CPu. In the latter area, the current study found VGF staining primarily in neuronal terminals distributed throughout the CPu but rarely in cell bodies, making it difficult to characterize a single location as the VGF side of SN projections (other techniques should be used to properly describe it).

This sentence must put into M+M: The VGF forms were identified by high performance liquid chromatography-mass spectrometry and western blot (WB), while VGF changes were determined by immunohistochemistry (IHC).

Answer: we have done it in the new version of the manuscript

Say clearly, in which structures the VGF C-t peptides are described (end of page 2), and where do you analysed them.

Answer: we have improved this part in the new version of the manuscript

2.2: dimethyl sulfoxide (DMSO, 1 µL) - what is the rational of intranigral DMSO? For me it is not clear, what control rats are: completely untreated or DMSO-injected? Please be very clear in that point.

Answer: In the present work, we used DMSO in continuity with our previous work (Bharatiya et al., 2020; doi: 10.1016/j.bbr.2020.112562) in which this solvent was used for the intranigral administration of fipronil. This means that the control rats of the locomotor activity experiments are DMSO-treated rats.

How long lasted the 1 microliter intranigral injection?

Answer: The injection lasted 1 minute. This information has been added in para 2.3 of the revised MS, as follows: "Rats were positioned in a Stoelting stereotaxic apparatus under isoflurane anaesthesia (1.5-2.0%) and a unilateral microinjection of DMSO (1 µL) or fipronil (25 µg/1 µL; purchased from Sigma Aldrich, Düsseldorf, Germany) was performed at the SN coordinates (AP: -5.3 mm; ML: -2.0 mm; DV: -8.0 mm) (Paxinos & Watson, 2004) over a period of 1 minute by using a 10 µL Hamilton microsyringe mounted on the holder of the Stoelting stereotaxic apparatus."

Please give a graph with a time line, indicating the exact days before and after the injections (i.e., when was what done exactly ?)

Answer: Thank you for this insightful suggestion. A timeline describing the sequence of the experimental procedures related to the rats used for the behavioral testing has been added to the revised MS (new Figure 1, so the older version of the figures now change in the new manuscript) together with its caption, as follows:
"Figure 1. Timeline of the experimental procedures performed with the cohort of rats dedicated to the behavioral testing of locomotor activity. After a period of ten days of acclimation and daily handling, each rat underwent one habituation session in the motility apparatus that lasted for two hours in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Twenty-four hours later, the rats were tested in order to obtain basal values of locomotor activity and build the experimental groups. Then, the day after, DMSO or fipronil microinjection into the substantia nigra was performed. Finally, after a recovery period of 15 days, each rat was individually tested for locomotor activity in order to assess potential effects of the fipronil intranigral microinjection and, thereafter, rats were perfused, and brains collected for the ex vivo IHC assays."

You said: … basal motor activity values, while 15 days after intranigral microinjection, each rat was tested for spontaneous locomotor activity …; what is the difference between basal motor activity and spontaneous locomotor activity?

Answer: When performing the behavioral evaluation of locomotor activity in lesioned animals it is routinary procedure of our Lab to carry out a first assessment of rats before performing the lesion (see, for instance, Angioni et al., 2016, doi: 10.1016/j.yhbeh.2016.05.012; Bharatiya et al., 2020, doi: 10.1016/j.bbr.2020.112562; Sanna et al., 2021, doi: 10.1016/j.brainres.2021.147705) in order to obtain information about the basal levels of activity for each rat. Then, we build the experimental groups counterbalancing them for the level of basal locomotor activity (i.e., the average of basal levels of activity between the new formed experimental groups will be very similar and should not differ statistically). This is done to avoid that the differences observed in the levels of locomotor activity after the lesioning procedure could be (in part) due to differences in basal activity of the rats that belong to the different experimental groups. We have now added details on this point in the text in order to make clearer this part of the procedure and have also described the experimental timeline of the rats used for the behavioral testing in a dedicated Figure (see also above).
As regards the expression "spontaneous locomotor activity", we traditionally refer to the activity of rats put in the experimental apparatus without any contingent experimental manipulation (pharmacological or other). In our case, the rats are injected with fipronil or the vehicle 15 days before the behavioral test. In this occasion, they are simply transferred from their home-cage to the experimental apparatus for the determination of their activity without any experimental manipulation in proximity of the behavioral test. For this reason, we refer to "spontaneous locomotor activity". However, we agree with the Referee that this point could be confounding for the reader and removed the term "spontaneous" throughout the revised MS.

Please give the name of the apparatus and the producer for monitoring the motor activity.

Answer: This information was already reported in the MS that describes the procedures and the apparatus for the determination of locomotor activity, that is a: "Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio)".

By the way: as I remember, spontaneous motor activity has no prior habituation.

Answer: In the present work, we were interested in evaluating the potential effects of the intranigral injection of the pesticide fipronil on locomotor activity to: 1) ensure that our behavioral model of fipronil-induced motor alterations previously characterized (see above and Bharatiya et al., 2020, doi:10.1016/j.bbr.2020.112562) was reproduced in the present experimentation; 2) investigate the potential involvement of VGF in the effects observed. Hence, we precisely adopted the same experimental conditions already used in our previous work for continuity (i.e., to make the present results directly comparable with the previous ones).
However, as a general comment, it should be noted that we always run habituation sessions before the experimental sessions when we are interested in evaluating the motor consequences of an experimental manipulation (see, for instance, Angioni et al., 2016, doi: 10.1016/j.yhbeh.2016.05.012; Bharatiya et al., 2020, doi:10.1016/j.bbr.2020.112562; Sanna et al., 2021; doi: 10.1016/j.brainres.2021.147705). This is done to avoid the influence on animal motor behavior of novelty factors related to the experimental procedure and the locomotor apparatus

during the experimental sessions and it is a commonly used procedure also by other Labs (see, for instance, Olmstead and Franklin, 1994, doi: 10.1016/0006-8993(94)90629-7).

2.4. why do you say antidody, although you used several of them

Answer: One is the antibody we generated and used for WB with untreated rats and for IHC through the rat groups, and it differs from the other two antibodies we purchased and used only for IHC in untreated rats to investigated whether they produced the same labelling as our antibody. We realized that using the word antigens could have made it less apparent, thus we changed the wording to "antigen" and improve this point in the new version of the manuscript.

You said: Both control and FPN-treated; what are control rats exactly?

Answer: We regret for not making it clear in the paper. As a result, in the revised MS, we refer to both DMSO and fipronil rats as "treated" (that is, the DMSO-treated rats served as controls for the fipronil-treated rats, as explained above). The remaining rats (n=4) will be referred to as "untreated rats" because they did not receive any surgical (or other) experimental procedures and were used only for VGF peptide characterization (WB and proteomic assays).

You said: Brains were rapidly removed, washed overnight in PBS containing 7% sucrose and 0.01% NaN3, oriented in aluminium foil moulds in cryo-embedding medium (Cocco et al., 2003) and frozen in melting Freon (cooled with liquid nitrogen). Coronal cryosections (10 µm) encompassing the entire SN obtained from the midbrain (from the section with AP≈-6.5 to the section with AP≈-4.5) and striatum were collected on poly-L-lysine-coated slides and stored in the vapour phase of a liquid nitrogen tank until use. After the fixation period, brains were embedded in the embedding medium described above and sectioned (at 10um) using a HM-560 cryomicrotome (Microm; Walldorf, Germany). My question: are there 2 different procedures?

Answer: we apologize for the error. We have deleted the sentence: "after the fixation period, brains were embedded in the embedding medium described above and sectioned (at 10um) using a HM-560 cryomicrotome (Microm; Walldorf, Germany)"

You said: density values for TH and VGF - that is incorrect: it must be the density of the binding of the respective antibodies.

Answer: we apologize for the error. We have changed the sentence in the new version of the manuscript.

WB analysis: what are untreated rats, how did you dissect the hypothalamus (which is quite difficult, at least for me) - was that dissected in fresh material? That is not mentioned as yet in the ms.

Answer To obtain samples, the technique described earlier (Noli et al, 2017) was followed. First, coronal slide samples were obtained from each FRESH brain (encompassing the areas of interest,) using a cooled rat brain matrix through razor blades, and then samples were obtained from the slides using punches of 3, 4, or 5 mm dimensions as appropriate, following the coordinates of the Paxinos Atlas of the rat brain (Paxinos and Watson, 2007). The hypothalamus was consistently

punctured around the third ventricle, which served as a reference point. In the updated manuscript, this section has been enlarged.

2.7. how many rats were used - at which time points? By the way, you must have used more than 16 rats or the number of samples is much too small to make a reliable result in the various parameters.

Answer As mentioned, Experiments with mass spectrometry were carried out on two distinct tissue extract pools of SN (from two rats) and CPu (from three rats) collected from the "untreated" control rat group. Because the goal of these investigations was to identify and characterize naturally occurring VGF peptides in SN and CPu, a qualitative top-down mass spectrometry technique was suited. The pooled tiny sample size is due to the use of qualitative rather than quantitative mass spectrometry analysis. Furthermore, only the IHC and behavioral data from the fipronil- and DMSO-treated rats were statistically analyzed (see above)

2.8. when you have results of 2 or 3 animals, how do you test for normality of the data distribution?

Answer: the section on "Statistical analysis" in paragraph 2.8 refers to IHC and behavioral analyses on locomotor activity and, as reported above, only DMSO- vs. fipronil-treated rats were included. This point has been made clear throughout the revised MS whenever appropriate.
Normal distribution of behavioral data has been checked by running the Shapiro-Wilk test. Normal distribution of ex vivo molecular assays has been checked by using the goodness-of-fit test. This information has been added in the revised version of the MS.

You said: FPN into the SN induced significant changes in spontaneous locomotor activity in our rats, in particular an increase in horizontal activity accompanied by a decrease in vertical activity - My comment: this is unusual and must be intensively discussed.

Answer We agree with the reviewer that usually horizontal and vertical activity changes follow the same direction after experimental manipulations (i.e., both parameters increase or decrease to some extent). However, there are examples in the literature in which this general trend is not observed under different conditions (see, for instance, Johnson, 1972; doi: 10.1007/BF01931862; Buzas et al., 2019, doi: 10.1016/j.brainres.2018.10.028).
There could be several reasons for the discrepancy between horizontal and vertical activity. One of them is that the two parameters quantify different aspects of motor behavior that involve partially different neural pathways. In fact, while horizontal activity (i.e., distance traveled in the horizontal plane) is usually considered an index of general motor activation and arousal and involves not only the activity of the dopaminergic nigrostriatal pathway but also that of the mesolimbic one (Sharp et al., 1987, doi: 10.1016/0006-8993(87)91416-8), vertical activity (usually expressed by the rearing behavior) is considered a more specific index of active exploration and has been specifically related to the nigrostriatal pathway and striatal dopamine function (see, for instance, Jicha and Salamone, 1991, doi: 10.1523/JNEUROSCI.11-12-03822.1991; Cousins et al., 1993, doi: 10.1016/0091-3057(93)90226-j).
Thus, it is possible that the lesion induced in our rats, inducing only a partial degeneration of the dopaminergic nigral neurons (approximately 40-50%), with a consequently relatively low decrease of dopamine in the striatum can lead to a decrease in vertical activity (more sensitive to partial

nigrostriatal damage) and, at the same time, to an imbalance in the activity of the pathways involved in the horizontal component of locomotor activity that lead to an increase other than a decrease in horizontal activity.
Support for this notion comes also from the observation that an increase in locomotor activity has been observed 6 days after treatment in rats with lesions of the nigrostriatal dopaminergic neurons induced by MPTP given at doses that produce a 50–60% decrease in striatal dopamine content (Ferro et al., 2005, doi: 10.1016/j.jneumeth.2005.04.005), although this difference was no more evident in the same rats after 18 days, pointing out the possibility that time-dependent alterations due to the time-course of the dopamine degeneration could be responsible of the different behavioral effects observed at different time-points. To note, in the same study 6-OHDA was not able to induce significant decreases in horizontal activity at both timepoints, though a tendency to decrease rearing behavior was reported after 6 days. Noteworthy, the most part of the studies that observed reductions in locomotor activity after the lesion of the nigrostriatal pathway, were conducted inducing bilateral lesions able to provoke up to 80% dopamine decrease (Deumens et al., 2002, doi: 10.1006/exnr.2002.7891) and often injecting the lesioning agent at the level of the medial forebrain bundle (MFB), thus inducing also the lesion of the dopaminergic mesolimbic pathway. For this reason, this kind of lesions is usually considered as a model of end-stage parkinsonism (see Yuan et al., 2005, doi: 10.1016/j.jneumeth.2004.10.004) and can differ in several aspects from other models where the lesion is of lower entity, as in our case. Further studies, in which the monitoring of rats injected with fipronil into the SN will last longer are needed to test this possibility.
Moreover, it should be taken into account that at variance from other lesioning agents that are able to induce highly selective lesions of the dopamine system when injected into the SN or in the MFB (such as 6-OHDA), fipronil could also act on other neurotransmitter systems, inducing less selective lesions able to imbalance the activity of the basal ganglia at a more generalized level (see, for instance, Angioni et al., 2016; doi: 10.1016/j.yhbeh.2016.05.012).
The Discussion of the revised version of the MS has been implemented accordingly.

You said in the discussion: FPN injection induced not only a reduction in TH … but also in VGF staining, in parallel with spontaneous changes in locomotor activity - My question: is this comparable with the literature on changes in motor activity? Please discuss deeply the effect of reduction of dopamine in the CPu and the changes on motor behavior.

Answer: Please, refer to the point discussed above regarding the relation between nigrostriatal dopamine and changes in locomotor activity.
As regards the potential involvement and role of VGF in the behavioral effects observed after fipronil treatment, it should be noted that the present is an explorative study that aims at investigating the presence of changes in VGF expression after fipronil-related TH depletion into the SN (as an index of nigrostriatal dopamine depletion, already reported in Bharatiya et al., 2020; doi: 10.1016/j.bbr.2020.112562) and in this view represents the first step of further investigations aimed at characterizing potential roles of the VGF fragments in the control of physiological and pathological motor behavior and exploration. Indeed, given to the scarcity of studies on the subject, the involvement of VGF in nigrostriatal circuits is not yet obvious, and more focalized research is required. The Discussion of the amended version of the MS has been carried out as planned.

Please work out the similarities and differences of your and the studies of Bharatiya´s group.

Answer Both the increase in the horizontal locomotor activity observed in the present study and the entity of the lesion induced in the TH immunoreactivity in the SN (by about 50%) closely resemble the findings reported and extensively discussed in our previous study, confirming the reproducibility of the model used (Bharatiya et al., 2020; doi: 10.1016/j.bbr.2020.112562).
At variance, we detected here a significant Time x Treatment interaction in the vertical activity data, indicating a slight, but significant, effect of the lesion also on the vertical component of motor behavior, that was not observed in our previous study. However, this difference is not more significant when analyzing the total scores of the 30 min test leading to hypothesize that under the conditions used in this and our previous study this behavioral effect can represent a borderline finding that deserves further attention in next investigations.
The Discussion of the revised version of the MS has been implemented accordingly.

You said: These results were also confirmed by the analyses of the total counts related to the whole 30 min test; accordingly, the t-test showed a significant difference between the two treatment groups for horizontal activity ($p = 0.005$) and a trend towards significance for vertical activity ($p = 0.065$). - My question: which parameters were changed in which way? Do you believe in trends?

Answer As reported in the Results of locomotor activity we observed a significant difference for the factor Treatment of the two-way ANOVA in the horizontal activity and a significant Treatment X Time interaction in the vertical activity when analyzing the 5 minutes time fractions. This kind of analysis can be informative in order to detect time-dependent changes in locomotor activity during the test, and the results obtained indicate that the lesion induced significant modifications in these two parameters when we compare the DMSO- and fipronil-treated rats. At variance, no significant differences were observed in the center time. However, we also analyzed the total counts of the 30 min experiment, that provides a global index of the level of activity for the whole test. In this case, we still detected a significant difference between the two groups in the horizontal activity but not the significance for vertical activity. For this reason, we initially reported the p value for vertical activity to show that it was very close to significance. However, we understand the issue raised by the Referee and removed this part from the revised version of the MS.
Accordingly, this section of Results has been modified to make clearer which parameters significantly differ between DMSO- and fipronil-treated rats.

Declaration of interests

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1 new

Click here to access/download;Figure;Fig, 1, time data.tif

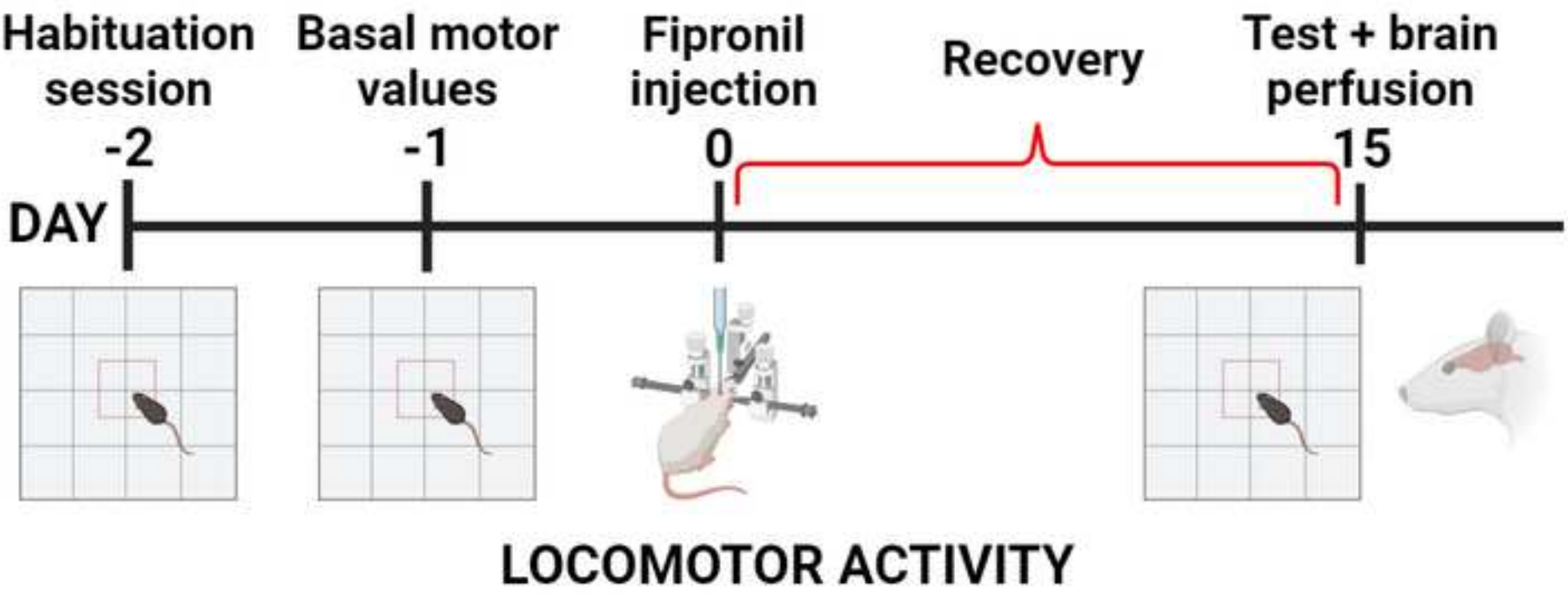


Figure 2 new

Click here to access/download;Figure;Fig, 2 wb fipronil NEW.tif

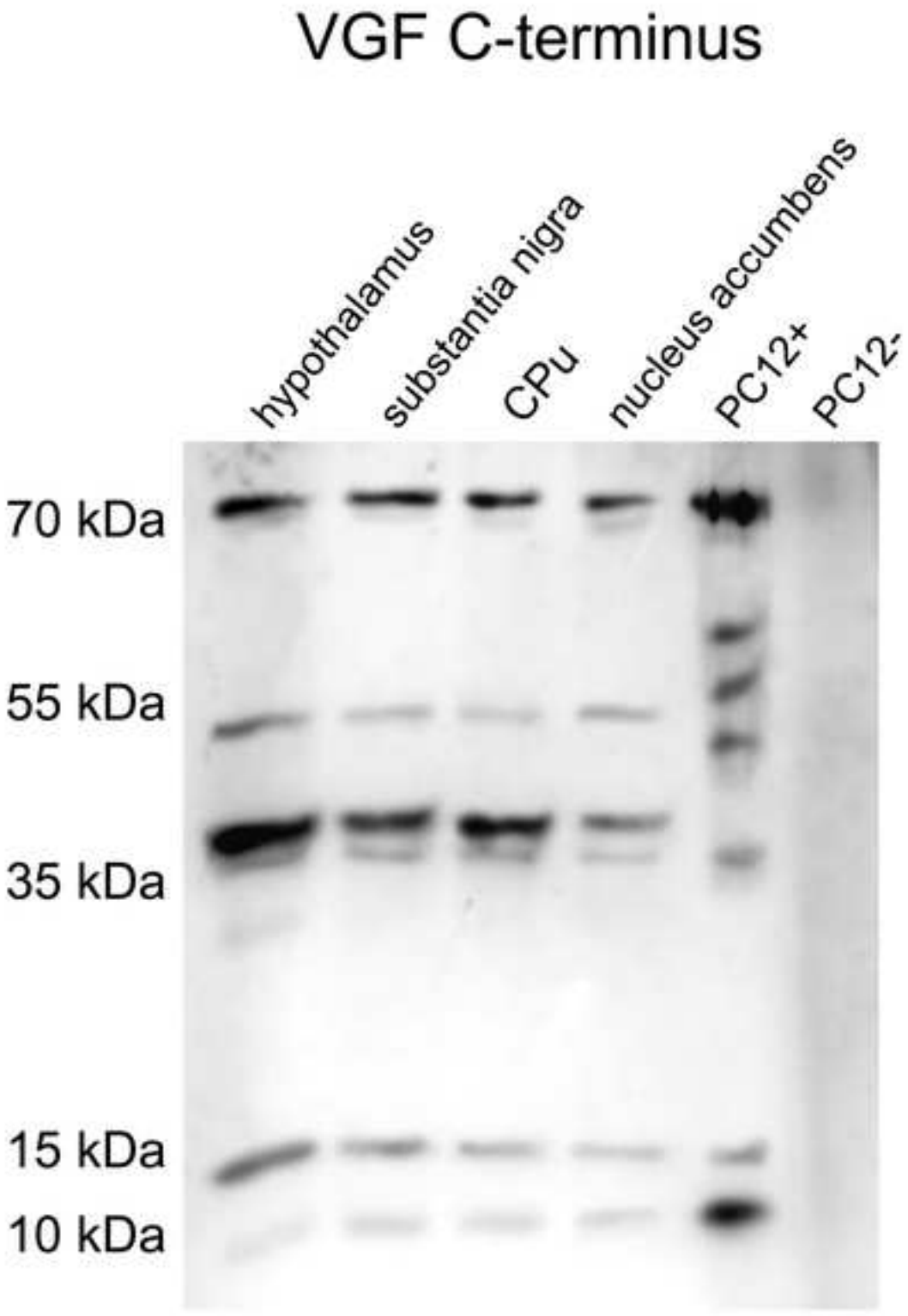


Figure 3

Click here to access/download;Figure;Fig, 2 HPLC.tif

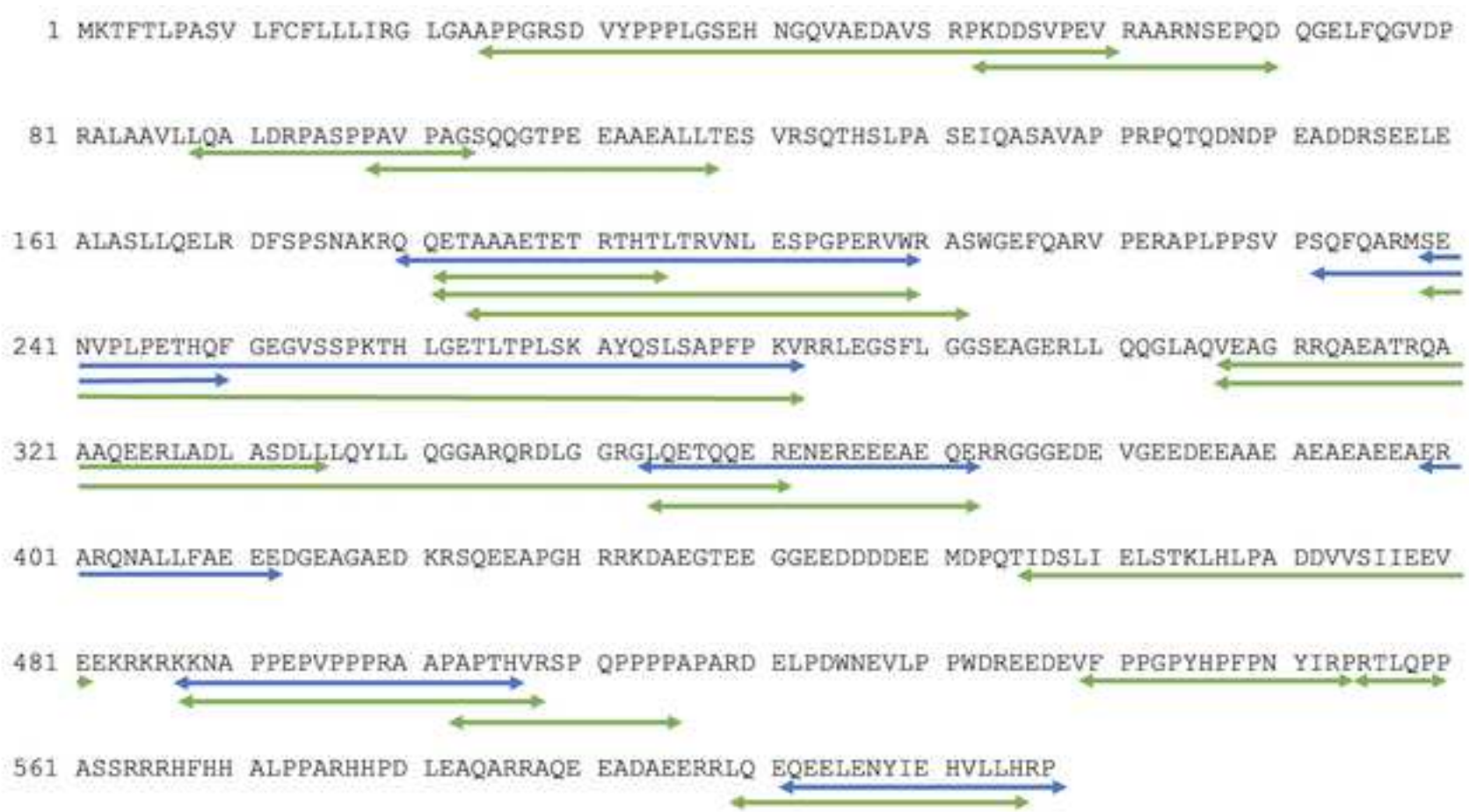


Figure 4

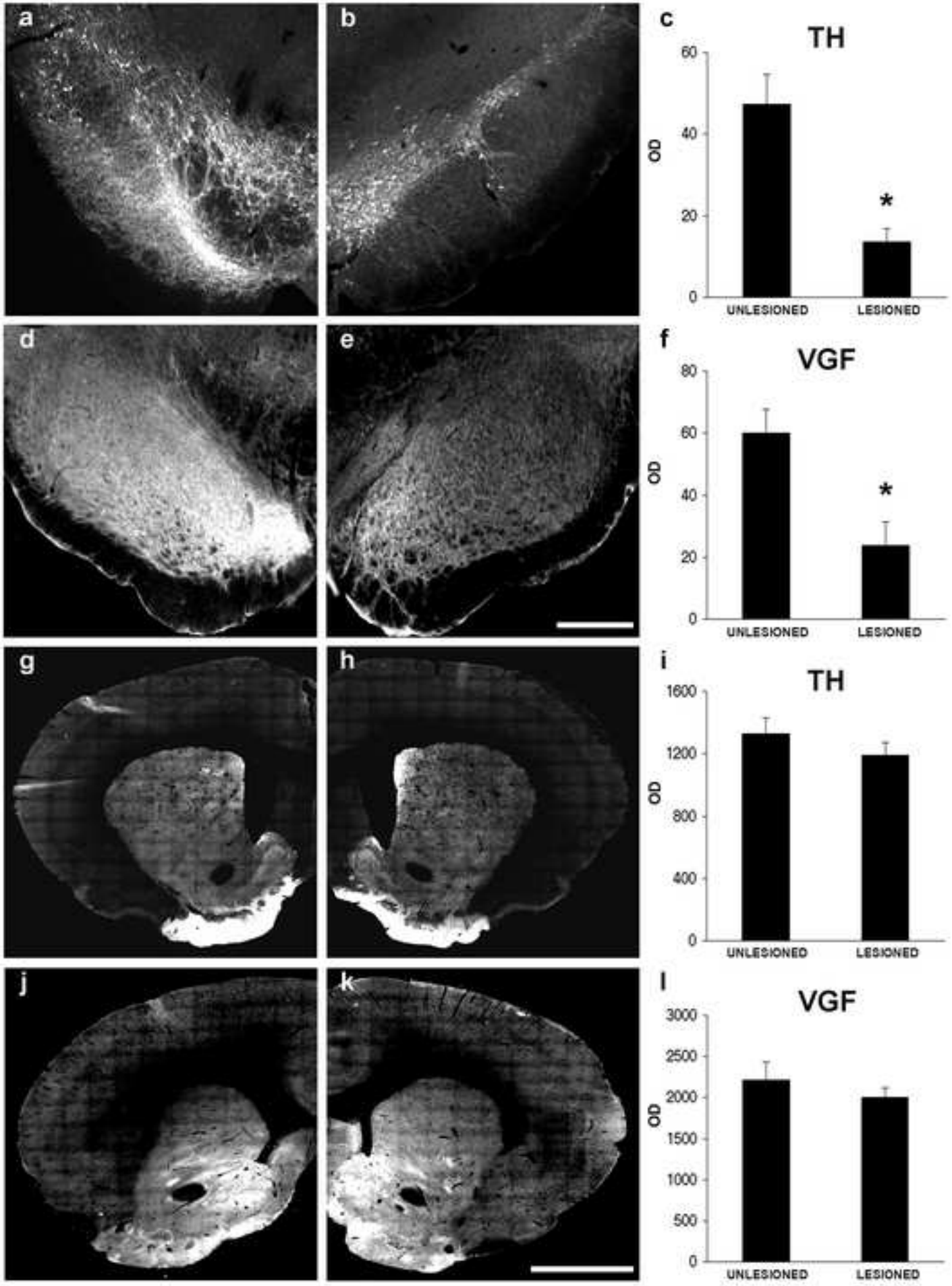


Figure 5

VGF sequence and antibodies

human MKALRLSASA LFC_LLLING LGAAPPGRPE AQPPPLSSEH KEPVAGDAVP GPKDGSAPEV RGARNSEPQD EGELFQGVDP
rat MKTFTLPASV LFCFLLLIRG LGAAPPGRSD VYPPPLGSEH NGQVAEDAVS RPKDDSVPEV RAARNSEPQD QGELFQGVDP

RALAAVLLQA LDRPASPPA_ PSGSQQGPEE EAAEALLTET VRSQTHSLPA PESPEPA_AP PRPQTPENGP EASDPSEELE
RALAAVLLQA LDRPASPPAV PAGSQQGTPE EAAEALLTES VRSQTHSLPA SEIQASAVAP PRPQTQDNDP EADDRSEELE

ALASLLQELR DFSPSSAKRQ QETAAAETET RTHTLTRVNL ESPGPERVWR ASWGEFQARV PERAPLPPPA PSQFQARMPD
ALASLLQELR DFSPSNAKRQ QETAAAETET RTHTLTRVNL ESPGPERVWR ASWGEFQARV PERAPLPPSV PSQFQARMSE

SGPLPETHKF GEGVSSPKTH LGEALAPLSK AYQGVAAPFP KARRPESALL GGSEAGERLL QQGLAQVEAG RRQAEATRQA
NVPLPETHQF GEGVSSPKTH LGETLTPLSK AYQSLSAPFP KVRRLEGSFL GGSEAGERLL QQGLAQVEAG RRQAEATRQA

AAQEERLADL ASDLLLQYLL QGGARQRGLG GRGLQEAAEE RESAREEEEA EQERRGGEE_ RVGEEDEEAA EAEAEAEEAE
AAQEERLADL ASDLLLQYLL QGGARQRDLG GRGLQETQQE RENER_EEEA EQERRGGGED EVGEEDEEAA EAEAEAEEAE

RARQNALLFA EEEDGEAGAE DKRSQEETPG HRRKEAEGTE EGGEEE_DDE EMDPQTIDSL IELSTKLHLP ADDVVSIIEE
RARQNALLFA EEEDGEAGAE DKRSQEEAPG HRRKDAEGTE EGGEEDDDDE EMDPQTIDSL IELSTKLHLP ADDVVSIIEE

VEEKRKRKKN APPEPVPPPR AAPAPTHVRS PQPPPPAPAP ARDELPDWNE VLPPWDREED EVYPPGPYHP FPNYIRPRTL
VEEKRKRKKN APPEPVPPPR AAPAPTHVRS PQPPPPAP_A _RDELPDWNE VLPPWDREED EVFPPGPYHP FPNYIRPRTL

QPPSALRRRH YHHALPPSRH YPGREAQARR AQEEAEAEER RLQEQEELEN YIEHVLLRRP
QPPASSRRRH FHHALPPARH HPDLEAQARR AQEEADAEER RLQEQEELEN YIEHVLLHRP

Ab1: raised to APARDELPDW NEVLPPWDRE EDEVYPPGPY HPFPNYIRPR TLQPPSALRR RHYHHALPPS RHYPGREAQA RRAQEEAEAE ERRLQEQEEL ENYIEHV (100aa)

Ab2: raised to EQEELENYIE HVLLRRP (17aa)

Ab3: raised to IEHVLLHRP (9aa)

Figure 6

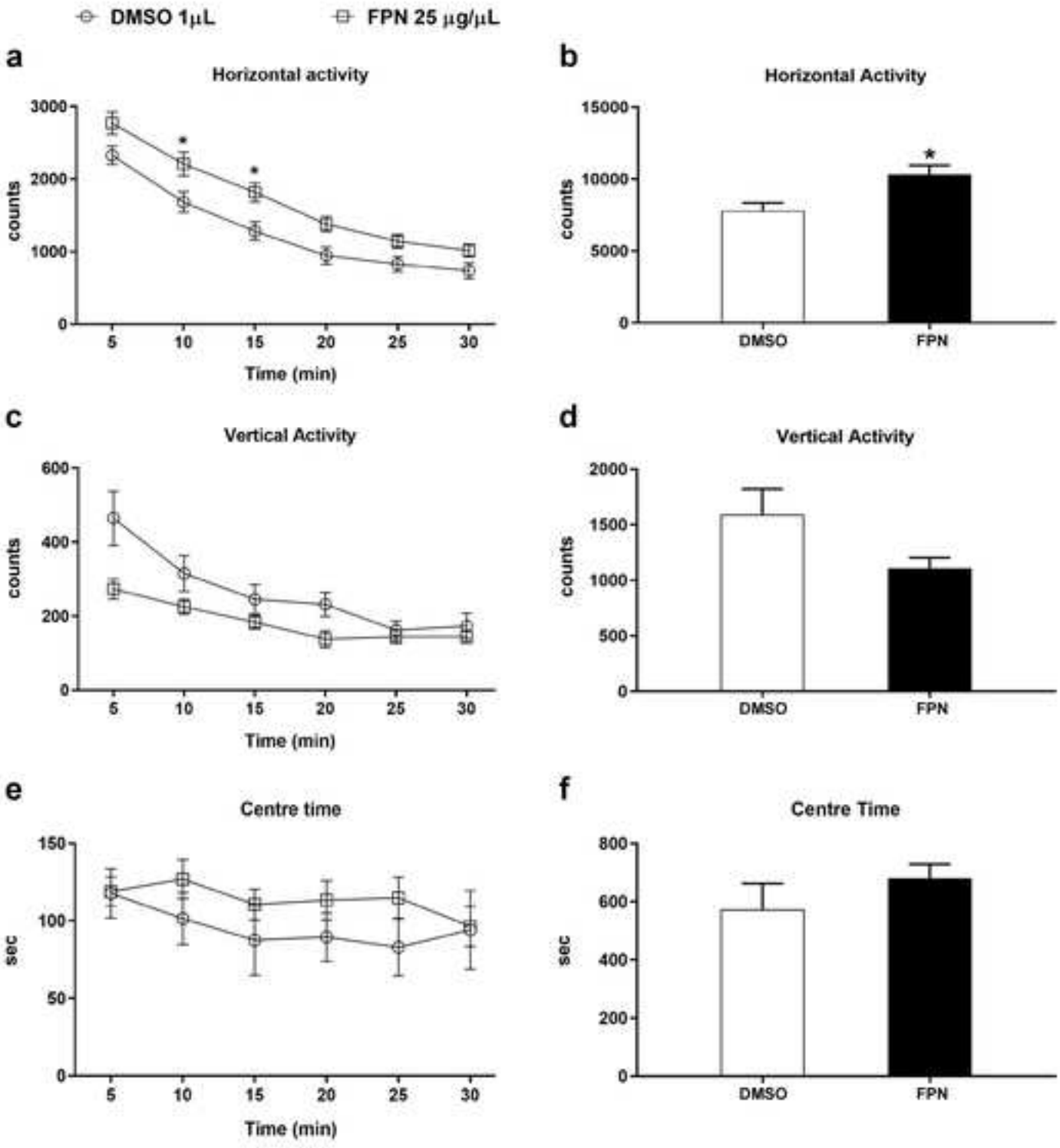


Table S1_A: Position, sequence and monoisotopic monocharged values of the naturally occurring peptides characterized by Top-down High-resolution MS/MS analysis from substantia nigra.

Position	Sequence	Monoisotopic monocharged values $[M+H]^+$
[180-209]	qQETAAAETETRTHTLTRVNLESPGPERVW	3390.66811
[232-250]	SQFQARMSENVPLPETHQF	2246.08745
[238-282]	MSENVPLPETHQFGEGVSSPKTHLGETLTPLSKAYQSLSAPFPKV	4836.47016
[353-372]	GLQETQQERENEREEEAEQE	2461.07004
[398-412]	AERARQNALLFAEEE	1746.86846
[487-507]	KKNAPPEPVPPPRAAPAPTHV	2171.21255
[601-617]	EQEELENYIEHVLLHRP	2148.07607

N-terminal q in the sequence denotes pyro glutamic acid;

Table S1_B. MS/MS characterization of the naturally occurring peptides from substantia nigra. For each peptide three tables are reported: peptide summary, fragment matches and fragment spectrum. In the fragment matches table red and blue values refer to b- and y-ion series respectively found in the fragment spectrum.

Sequence: QQETAAAETETRTHTLTRVNLESPGPERVW, Q1-Gln->pyro-Glu (-17.02655 Da)
Charge: +4, Monoisotopic m/z: 848.42249 Da (+0.17 mmu/+0.2 ppm), MH+: 3390.66811 Da, RT: 22.66 min,
Identified with: Sequest HT (v1.3); XCorr:5.96, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

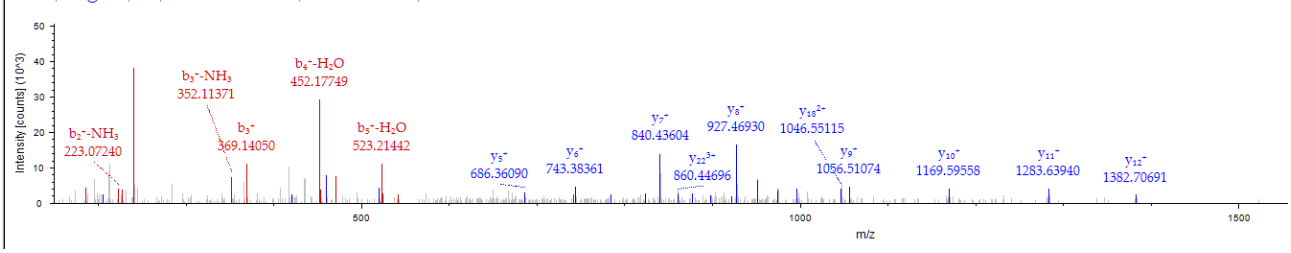
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	b4+	Seq.	y+	y2+	y3+	y4+	#2
1	112.03931	56.52329	38.01795	28.76528	Q-Gln->pyr...					30
2	240.09789	120.55258	80.70415	60.77993	Q	3279.63540	1640.32134	1093.88332	820.66431	29
3	369.14049	185.07388	123.71835	93.04058	E	3151.57682	1576.29205	1051.19712	788.64966	28
4	470.18817	235.59772	157.40091	118.30250	T	3022.53422	1511.77075	1008.18292	756.38901	27
5	541.22529	271.11628	181.07995	136.06178	A	2921.48654	1461.24691	974.50036	731.12709	26
6	612.26241	306.63484	204.75899	153.82106	A	2850.44942	1425.72835	950.82132	713.36781	25
7	683.29953	342.15340	228.43803	171.58034	A	2779.41230	1390.20979	927.14228	695.60853	24
8	812.34213	406.67470	271.45223	203.84099	E	2708.37518	1354.69123	903.46324	677.84925	23
9	913.38981	457.19854	305.13479	229.10291	T	2579.33258	1290.16993	860.44904	645.58860	22
10	1042.43241	521.71984	348.14899	261.36356	E	2478.28490	1239.64609	826.76648	620.32668	21
11	1143.48009	572.24368	381.83155	286.62548	T	2349.24230	1175.12479	783.75228	588.06603	20
12	1299.58121	650.29424	433.86525	325.65076	R	2248.19462	1124.60095	750.06972	562.80411	19
13	1400.62889	700.81808	467.54781	350.91268	T	2092.09350	1046.55039	698.03602	523.77883	18
14	1537.68780	769.34754	513.23412	385.17741	H	1991.04582	996.02655	664.35346	498.51691	17
15	1638.73548	819.87138	546.91668	410.43933	T	1853.98691	927.49709	618.66715	464.25219	16
16	1751.81955	876.41341	584.61137	438.71034	L	1752.93923	876.97325	584.98459	438.99027	15
17	1852.86723	926.93725	618.29393	463.97226	T	1639.85516	820.43122	547.28990	410.71925	14
18	2008.96835	1004.98781	670.32763	502.99754	R	1538.80748	769.90738	513.60734	385.45733	13
19	2108.03677	1054.52202	703.35044	527.76465	V	1382.70636	691.85682	461.57364	346.43205	12
20	2222.07970	1111.54349	741.36475	556.27538	N	1283.63794	642.32261	428.55083	321.66494	11
21	2335.16377	1168.08552	779.05944	584.54640	L	1169.59501	585.30114	390.53652	293.15421	10
22	2464.20637	1232.60682	822.07364	616.80705	E	1056.51094	528.75911	352.84183	264.88319	9
23	2551.23840	1276.12284	851.08432	638.56506	S	927.46834	464.23781	309.82763	232.62254	8
24	2648.29117	1324.64922	883.43524	662.82825	P	840.43631	420.72179	280.81695	210.86454	7
25	2705.31264	1353.15996	902.44240	677.08362	G	743.38354	372.19541	248.46603	186.60134	6
26	2802.36541	1401.68634	934.79332	701.34681	P	686.36207	343.68467	229.45887	172.34598	5
27	2931.40801	1466.20764	977.80752	733.60746	E	589.30930	295.15829	197.10795	148.08278	4
28	3087.50913	1544.25820	1029.84123	772.63274	R	460.26670	230.63699	154.09375	115.82213	3
29	3186.57755	1593.79241	1062.86403	797.39984	V	304.16558	152.58643	102.06004	76.79685	2
30					W	205.09716	103.05222	69.03724	52.02975	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_1.raw #2982 RT: 22.66
FTMS, HCD@40.00, z=+4, Mono m/z=848.42249 Da, MH+=3390.66811 Da, Match Tol.=0.02 Da



Sequence: SQFQARMSENVPLPETHQF, Charge: +2, Monoisotopic m/z: 1123.54736 Da (+10.74 mmu/+9.56 ppm), MH+: 2246.08745 Da, RT: 22.40 min,
Identified with: Sequest HT (v1.3); XCorr:0.85, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

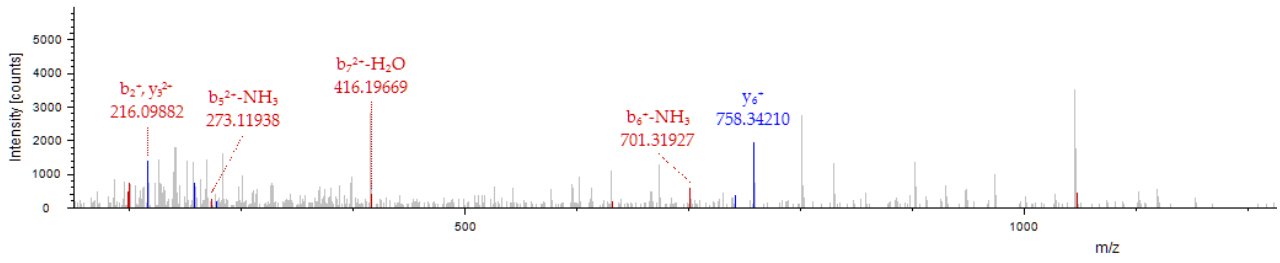
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	88.03931	44.52329	S			19
2	216.09789	108.55258	Q	2159.03394	1080.02061	18
3	363.16631	182.08679	F	2030.97536	1015.99132	17
4	491.22489	246.11608	Q	1883.90694	942.45711	16
5	562.26201	281.63464	A	1755.84836	878.42782	15
6	718.36313	359.68520	R	1684.81124	842.90926	14
7	849.40363	425.20545	M	1528.71012	764.85870	13
8	936.43566	468.72147	S	1397.66962	699.33845	12
9	1065.47826	533.24277	E	1310.63759	655.82243	11
10	1179.52119	590.26423	N	1181.59499	591.30113	10
11	1278.58961	639.79844	V	1067.55206	534.27967	9
12	1375.64238	688.32483	P	968.48364	484.74546	8
13	1488.72645	744.86686	L	871.43087	436.21907	7
14	1585.77922	793.39325	P	758.34680	379.67704	6
15	1714.82182	857.91455	E	661.29403	331.15065	5
16	1815.86950	908.43839	T	532.25143	266.62935	4
17	1952.92841	976.96784	H	431.20375	216.10551	3
18	2080.98699	1040.99713	Q	294.14484	147.57606	2
19			F	166.08626	83.54677	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_1.raw #2945 RT: 22.40
FTMS, HCD@40.00, z=+2, Mono m/z=1123.54736 Da, MH+=2246.08745 Da, Match Tol.=0.02 Da



Sequence: MSENVPLPETHQFGEGVSSPKTHLGETLTPLSKAYQSLSAPFPKV, Charge: +5, Monoisotopic m/z: 968.09985 Da (+2.93 mmu/+3.03 ppm), MH+: 4836.47016 Da, RT: 32.26 min,
Identified with: Sequest HT (v1.3); XCorr:3.32, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

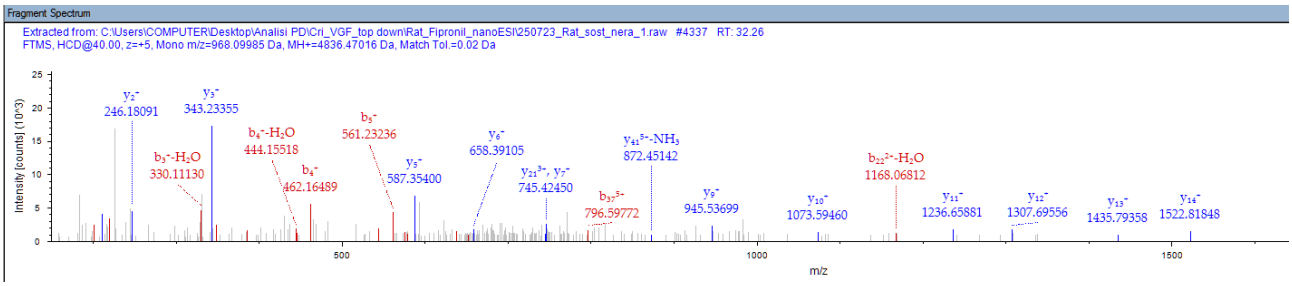
Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	b^{5+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	y^{5+}	#2
1	132.04778	66.52753	44.68744	33.76740	27.21538	M						45
2	219.07981	110.04354	73.69812	55.52541	44.62178	S	4705.41500	2353.21114	1569.14318	1177.10921	941.88882	44
3	348.12241	174.56484	116.71232	87.78606	70.43030	E	4618.38297	2309.69512	1540.13251	1155.35120	924.48242	43
4	462.16534	231.58631	154.72663	116.29679	93.23889	N	4489.34037	2245.17382	1497.11831	1123.09055	898.67390	42
5	561.23376	281.12052	187.74944	141.06390	113.05257	V	4375.29744	2188.15236	1459.10400	1094.57982	875.86531	41
6	658.28653	329.64690	220.10036	165.32709	132.46313	P	4276.22902	2138.61815	1426.08119	1069.81271	856.05163	40
7	771.37060	386.18894	257.79505	193.59811	155.07994	L	4179.17625	2090.09176	1393.73027	1045.54952	836.64107	39
8	868.42337	434.71532	290.14597	217.86130	174.49049	P	4066.09218	2033.54973	1356.03558	1017.27850	814.02426	38
9	997.46597	499.23662	333.16017	250.12195	200.29901	E	3969.03941	1985.02334	1323.68465	993.01531	794.61370	37
10	1098.51365	549.76046	366.84273	275.38387	220.50855	T	3839.99681	1920.50204	1280.67045	960.75466	768.80518	36
11	1235.57256	618.28992	412.52904	309.64860	247.92033	H	3738.94913	1869.97820	1246.98789	935.49274	748.59565	35
12	1363.63114	682.31921	455.21523	341.66324	273.53205	Q	3601.89022	1801.44875	1201.30159	901.22801	721.18387	34
13	1510.69956	755.85342	504.23804	378.43035	302.94573	F	3473.83164	1737.41946	1158.61540	869.21337	695.57215	33
14	1567.72103	784.36415	523.24519	392.68571	314.35003	G	3326.76322	1663.88525	1109.59259	832.44626	666.15847	32
15	1696.76363	848.88545	566.25939	424.94636	340.15855	E	3269.74175	1635.37451	1090.58543	818.19090	654.75417	31
16	1753.78510	877.39619	585.26655	439.20173	351.56284	G	3140.69915	1570.85321	1047.57123	785.93025	628.94565	30
17	1852.85352	926.93040	618.28936	463.96884	371.37652	V	3083.67768	1542.34248	1028.56408	771.67488	617.54136	29
18	1939.88555	970.44641	647.30003	485.72684	388.78293	S	2984.60926	1492.80827	995.54127	746.90777	597.72767	28
19	2026.91758	1013.96243	676.31071	507.48485	406.18934	S	2897.57723	1449.29225	966.53059	725.14977	580.32127	27
20	2123.97035	1062.48881	708.66163	531.74804	425.59989	P	2810.54520	1405.77624	937.51992	703.39176	562.91486	26
21	2252.06532	1126.53630	751.35996	563.77179	451.21888	K	2713.49243	1357.24985	905.16899	679.12857	543.50431	25
22	2353.11300	1177.06014	785.04252	589.03371	471.42842	T	2585.39746	1293.20237	862.47067	647.10482	517.88531	24
23	2490.17191	1245.58959	830.72882	623.29843	498.84020	H	2484.34978	1242.67853	828.78811	621.84290	497.67578	23
24	2603.25598	1302.13163	868.42351	651.56945	521.45702	L	2347.29087	1174.14907	783.10181	587.57818	470.26400	22
25	2660.27745	1330.64236	887.43067	665.82482	532.86131	G	2234.20680	1117.60704	745.40712	559.30716	447.64718	21
26	2789.32005	1395.16366	930.44487	698.08547	558.66983	E	2177.18533	1089.09630	726.39996	545.05179	436.24289	20
27	2890.36773	1445.68750	964.12743	723.34739	578.87937	T	2048.14273	1024.57500	683.38576	512.79114	410.43437	19
28	3003.45180	1502.22954	1001.82212	751.61841	601.49618	L	1947.09505	974.05116	649.70320	487.52922	390.22483	18
29	3104.49948	1552.75338	1035.50468	776.88033	621.70572	T	1834.01098	917.50913	612.00851	459.25820	367.60802	17
30	3201.55225	1601.27976	1067.85560	801.14352	641.11627	P	1732.96330	866.98529	578.32595	433.99628	347.39848	16
31	3314.63632	1657.82180	1105.55029	829.41454	663.73308	L	1635.91053	818.45890	545.97503	409.73309	327.98793	15
32	3401.66835	1701.33781	1134.56097	851.17254	681.13949	S	1522.82646	761.91687	508.28034	381.46207	305.37111	14
33	3529.76332	1765.38530	1177.25929	883.19629	706.75848	K	1435.79443	718.40085	479.26966	359.70407	287.96471	13
34	3600.80044	1800.90386	1200.93833	900.95557	720.96591	A	1307.69946	654.35337	436.57134	327.68032	262.34571	12
35	3763.86376	1882.43552	1255.29277	941.72140	753.57857	Y	1236.66234	618.83481	412.89230	309.92104	248.13829	11
36	3891.92234	1946.46481	1297.97896	973.73604	779.19029	Q	1073.59902	537.30315	358.53786	269.15521	215.52563	10
37	3978.95437	1989.98082	1326.98964	995.49405	796.59669	S	945.54044	473.27386	315.85166	237.14057	189.91391	9
38	4092.03844	2046.52286	1364.68433	1023.76507	819.21351	L	858.50841	429.75784	286.84099	215.38256	172.50750	8
39	4179.07047	2090.03887	1393.69501	1045.52307	836.61991	S	745.42434	373.21581	249.14630	187.11154	149.89069	7
40	4250.10759	2125.55743	1417.37405	1063.28235	850.82734	A	658.39231	329.69979	220.13562	165.35354	132.48428	6
41	4347.16036	2174.08382	1449.72497	1087.54555	870.23789	P	587.35519	294.18123	196.45658	147.59426	118.27686	5
42	4494.22878	2247.61803	1498.74778	1124.31265	899.65158	F	490.30242	245.65485	164.10566	123.33106	98.86631	4
43	4591.28155	2296.14441	1531.09870	1148.57584	919.06213	P	343.23400	172.12064	115.08285	86.56396	69.45262	3
44	4719.37652	2360.19190	1573.79702	1180.59959	944.68112	K	246.18123	123.59425	82.73193	62.30077	50.04207	2
45						V	118.08626	59.54677	40.03360	30.27702	24.42307	1

Fragment Spectrum



Sequence: GLQETQQERENEREEEAEQE, Charge: +3, Monoisotopic m/z: 821.02820 Da (-0.34 mmu/-0.42 ppm), MH+: 2461.07004 Da, RT: 13.73 min,
Identified with: Sequest HT (v1.3); XCorr:3.57, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

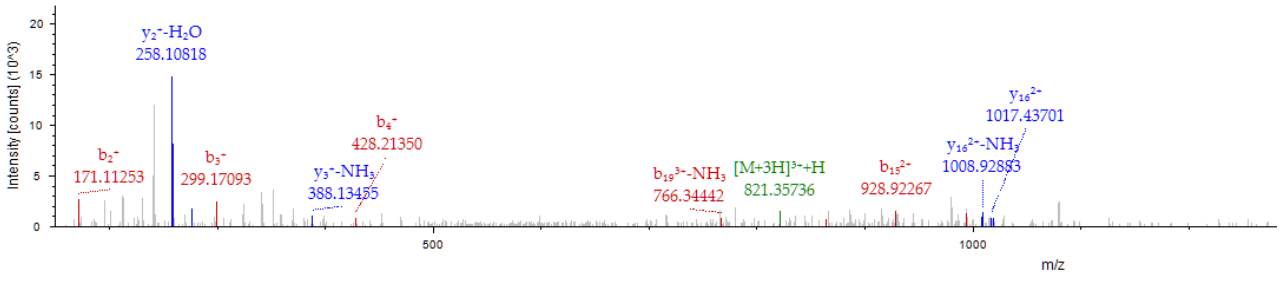
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	Seq.	y^+	y^{2+}	y^{3+}	#2
1	58.02875	29.51801	20.01443	G				20
2	171.11282	86.06005	57.70912	L	2404.04960	1202.52844	802.02138	19
3	299.17140	150.08934	100.39532	Q	2290.96553	1145.98640	764.32669	18
4	428.21400	214.61064	143.40952	E	2162.90695	1081.95711	721.64050	17
5	529.26168	265.13448	177.09208	T	2033.86435	1017.43581	678.62630	16
6	657.32026	329.16377	219.77827	Q	1932.81667	966.91197	644.94374	15
7	785.37884	393.19306	262.46446	Q	1804.75809	902.88268	602.25755	14
8	914.42144	457.71436	305.47866	E	1676.69951	838.85339	559.57135	13
9	1070.52256	535.76492	357.51237	R	1547.65691	774.33209	516.55715	12
10	1199.56516	600.28622	400.52657	E	1391.55579	696.28153	464.52345	11
11	1313.60809	657.30768	438.54088	N	1262.51319	631.76023	421.50925	10
12	1442.65069	721.82898	481.55508	E	1148.47026	574.73877	383.49494	9
13	1598.75181	799.87954	533.58879	R	1019.42766	510.21747	340.48074	8
14	1727.79441	864.40084	576.60299	E	863.32654	432.16691	288.44703	7
15	1856.83701	928.92214	619.61719	E	734.28394	367.64561	245.43283	6
16	1985.87961	993.44344	662.63139	E	605.24134	303.12431	202.41863	5
17	2056.91673	1028.96200	686.31043	A	476.19874	238.60301	159.40443	4
18	2185.95933	1093.48330	729.32463	E	405.16162	203.08445	135.72539	3
19	2314.01791	1157.51259	772.01082	Q	276.11902	138.56315	92.71119	2
20				E	148.06044	74.53386	50.02500	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_1.raw #1678 RT: 13.73
FTMS, HCD@40.00, z=+3, Mono m/z=821.02820 Da, MH+=2461.07004 Da, Match Tol.=0.02 Da



Sequence: AERARQNALLFAEEE, Charge: +2, Monoisotopic m/z: 873.93787 Da (-4.29 mmu/-4.91 ppm), MH+: 1746.86846 Da, RT: 17.03 min,
Identified with: Sequest HT (v1.3); XCorr:0.77, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

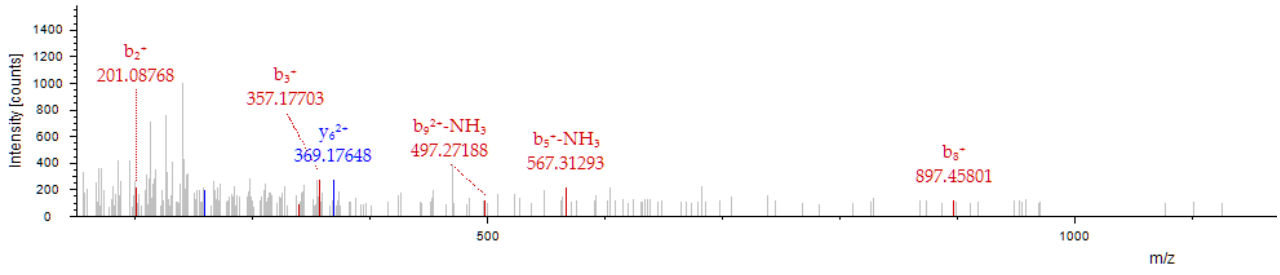
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	72.04440	36.52584	A			15
2	201.08700	101.04714	E	1675.83991	838.42359	14
3	357.18812	179.09770	R	1546.79731	773.90229	13
4	428.22524	214.61626	A	1390.69619	695.85173	12
5	584.32636	292.66682	R	1319.65907	660.33317	11
6	712.38494	356.69611	Q	1163.55795	582.28261	10
7	826.42787	413.71757	N	1035.49937	518.25332	9
8	897.46499	449.23613	A	921.45644	461.23186	8
9	1010.54906	505.77817	L	850.41932	425.71330	7
10	1123.63313	562.32020	L	737.33525	369.17126	6
11	1270.70155	635.85441	F	624.25118	312.62923	5
12	1341.73867	671.37297	A	477.18276	239.09502	4
13	1470.78127	735.89427	E	406.14564	203.57646	3
14	1599.82387	800.41557	E	277.10304	139.05516	2
15			E	148.06044	74.53386	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_1.raw #2209 RT: 17.03
FTMS, HCD@40.00, z=+2, Mono m/z=873.93787 Da, MH+=1746.86846 Da, Match Tol.=0.02 Da



Sequence: KKNAPPEPVPPPRAAPAPTHV, Charge: +4, Monoisotopic m/z: 543.55859 Da (+1.01 mmu/+1.86 ppm), MH+: 2171.21255 Da, RT: 14.46 min,
Identified with: Sequest HT (v1.3); XCorr:3.53, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

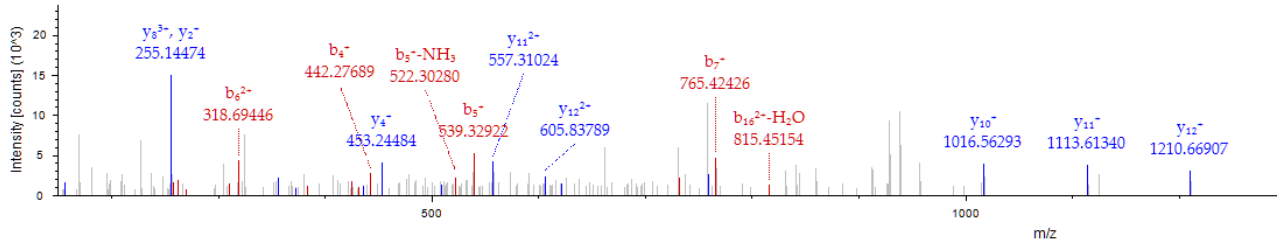
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	#2
1	129.10225	65.05476	43.70560	33.03102	K					21
2	257.19722	129.10225	86.40392	65.05476	K	2043.11353	1022.06040	681.70936	511.53384	20
3	371.24015	186.12371	124.41823	93.56549	N	1915.01856	958.01292	639.01104	479.51010	19
4	442.27727	221.64227	148.09727	111.32477	A	1800.97563	900.99145	600.99673	450.99937	18
5	539.33004	270.16866	180.44820	135.58797	P	1729.93851	865.47289	577.31769	433.24009	17
6	636.38281	318.69504	212.79912	159.85116	P	1632.88574	816.94651	544.96676	408.97689	16
7	765.42541	383.21634	255.81332	192.11181	E	1535.83297	768.42012	512.61584	384.71370	15
8	862.47818	431.74273	288.16424	216.37500	P	1406.79037	703.89882	469.60164	352.45305	14
9	961.54660	481.27694	321.18705	241.14211	V	1309.73760	655.37244	437.25072	328.18986	13
10	1058.59937	529.80332	353.53797	265.40530	P	1210.66918	605.83823	404.22791	303.42275	12
11	1155.65214	578.32971	385.88890	289.66849	P	1113.61641	557.31184	371.87699	279.15956	11
12	1252.70491	626.85609	418.23982	313.93168	P	1016.56364	508.78546	339.52606	254.89637	10
13	1408.80603	704.90665	470.27353	352.95696	R	919.51087	460.25907	307.17514	230.63318	9
14	1479.84315	740.42521	493.95257	370.71624	A	763.40975	382.20851	255.14143	191.60790	8
15	1550.88027	775.94377	517.63161	388.47552	A	692.37263	346.68995	231.46239	173.84862	7
16	1647.93304	824.47016	549.98253	412.73872	P	621.33551	311.17139	207.78335	156.08934	6
17	1718.97016	859.98872	573.66157	430.49800	A	524.28274	262.64501	175.43243	131.82614	5
18	1816.02293	908.51510	606.01249	454.76119	P	453.24562	227.12645	151.75339	114.06686	4
19	1917.07061	959.03894	639.69505	480.02311	T	356.19285	178.60006	119.40247	89.80367	3
20	2054.12952	1027.56840	685.38136	514.28784	H	255.14517	128.07622	85.71991	64.54175	2
21					V	118.08626	59.54677	40.03360	30.27702	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_2.raw #1801 RT: 14.46
FTMS, HCD@40.00, z=+4, Mono m/z=543.55859 Da, MH+=2171.21255 Da, Match Tol.=0.02 Da



Sequence: EQEELENYIEHVLLHRP, Charge: +4, Monoisotopic m/z: 537.77448 Da (+1 mmu/+1.86 ppm), MH+: 2148.07607 Da, RT: 36.46 min,
Identified with: Sequest HT (v1.3); XCorr:2.49, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	#2
1	130.04988	65.52858	44.02148	33.26793	E					17
2	258.10846	129.55787	86.70767	65.28257	Q	2019.02948	1010.01838	673.68134	505.51283	16
3	387.15106	194.07917	129.72187	97.54322	E	1890.97090	945.98909	630.99515	473.49818	15
4	516.19366	258.60047	172.73607	129.80387	E	1761.92830	881.46779	587.98095	441.23753	14
5	629.27773	315.14250	210.43076	158.07489	L	1632.88570	816.94649	544.96675	408.97688	13
6	758.32033	379.66380	253.44496	190.33554	E	1519.80163	760.40445	507.27206	380.70587	12
7	872.36326	436.68527	291.45927	218.84627	N	1390.75903	695.88315	464.25786	348.44522	11
8	1035.42658	518.21693	345.81371	259.61210	Y	1276.71610	638.86169	426.24355	319.93448	10
9	1148.51065	574.75896	383.50840	287.88312	I	1113.65278	557.33003	371.88911	279.16865	9
10	1277.55325	639.28026	426.52260	320.14377	E	1000.56871	500.78799	334.19442	250.89764	8
11	1414.61216	707.80972	472.20890	354.40850	H	871.52611	436.26669	291.18022	218.63699	7
12	1513.68058	757.34393	505.23171	379.17560	V	734.46720	367.73724	245.49392	184.37226	6
13	1626.76465	813.88596	542.92640	407.44662	L	635.39878	318.20303	212.47111	159.60515	5
14	1739.84872	870.42800	580.62109	435.71764	L	522.31471	261.66099	174.77642	131.33414	4
15	1876.90763	938.95745	626.30739	469.98236	H	409.23064	205.11896	137.08173	103.06312	3
16	2033.00875	1017.00801	678.34110	509.00764	R	272.17173	136.58950	91.39543	68.79839	2
17					P	116.07061	58.53894	39.36172	29.77311	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_sost_nera_2.raw #5046 RT: 36.46
FTMS, HCD@40.00, z=+4, Mono m/z=537.77448 Da, MH+=2148.07607 Da, Match Tol.=0.02 Da

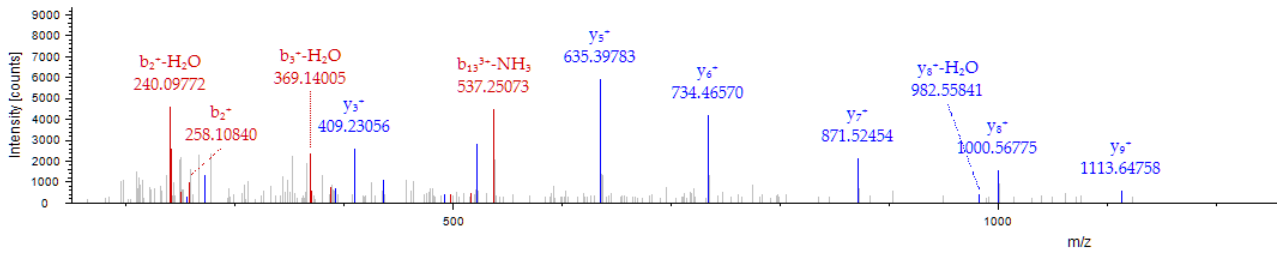


Table S2_A: Position, sequence and monoisotopic monocharged values of the naturally occurring peptides characterized by Top-down High-resolution MS/MS analysis from striatum.

Position	Sequence	Monoisotopic monocharged values $[M+H]^+$
[24-60]	APPGRSDVYPPPLGSEHNGQVAEDAVSRPKDDSVPEV	3868.88532
[52-69]	PKDDSVPEVRAARNSEPQ	1994.98747
[88-103]	LQALDRPASPPAVPAG	1559.85624
[98-117]	PAVPAGSQQGTPEEAAEALL	1934.97515
[180-194]	QQETAAAETETRTHT	1656.75732
[180-209]	QQETAAAETETRTHTLTRVNLESPGPERVW	3390.69130
[183-211]	TAAAETETRTHTLTRVNLESPGPERVWRA	3249.67617
[238-282]	MSENVPLPETHQFGEGVSSPKTHLGETLTPLSKAYQSLSAPFPKV	4836.46894
[306-335]	QVEAGRRQAEATRQAAAQEERLADLASDLL	3249.67617
[306-361]	QVEAGRRQAEATRQAAAQEERLADLASDLLLQYLLQGGARQRDLGGRGLQETQQER	6217.20312
[353-372]	GLQETQQERENEREEEAEQE	2461.07535
[455-482]	TIDSLIELSTKLHLPADDVVSIIEEVEE	3106.66445
[487-507]	KKNAPPEPVPPPRAAPAPTHV	2171.21474
[502-515]	PAPTHVRSPQPPPP	1477.78562
[539-553]	VFPPGPYHPFPNYIR	1800.91581
[554-559]	PRTLQP	710.43205
[599-611]	LQEQEELENYIEH	1973.75920

Table S2_B. MS/MS characterization of the naturally occurring peptides from striatum. For each peptide three tables are reported: peptide summary, fragment matches and fragment spectrum. In the fragment matches table red and blue values refer to the b- and y-ion series respectively found in the fragment spectrum.

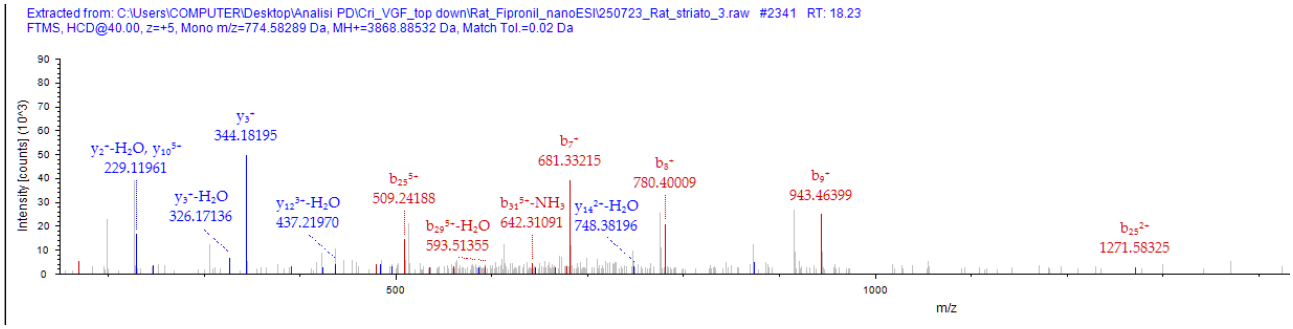
Sequence: APPGRSDVYPPPLGSEHNGQVAEDAVSRPKDDSVPEV, Charge: +5, Monoisotopic m/z: 774.58289 Da (+2.3 mmu/+2.97 ppm), MH+: 3868.88532 Da, R
Identified with: Sequest HT (v1.3); XCorr:2.24, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	b^{5+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	y^{5+}	#2
1	72.04440	36.52584	24.68632	18.76656	15.21470	A						37
2	169.09717	85.05222	57.03724	43.02975	34.62525	P	3797.83672	1899.42200	1266.61709	950.21464	760.37317	36
3	266.14994	133.57861	89.38816	67.29294	54.03581	P	3700.78395	1850.89561	1234.26617	925.95145	740.96261	35
4	323.17141	162.08934	108.39532	81.54831	65.44010	G	3603.73118	1802.36923	1201.91524	901.68825	721.55206	34
5	479.27253	240.13990	160.42903	120.57359	96.66033	R	3546.70971	1773.85849	1182.90809	887.43289	710.14776	33
6	566.30456	283.65592	189.43970	142.33160	114.06673	S	3390.60859	1695.80793	1130.87438	848.40761	678.92754	32
7	681.33151	341.16939	227.78202	171.08833	137.07212	D	3303.57656	1652.29192	1101.86370	826.64960	661.52113	31
8	780.39993	390.70360	260.80483	195.85544	156.88581	V	3188.54961	1594.77844	1063.52139	797.89286	638.51574	30
9	943.46325	472.23526	315.15927	236.62127	189.49847	Y	3089.48119	1545.24423	1030.49858	773.12576	618.70206	29
10	1040.51602	520.76165	347.51019	260.88446	208.90902	P	2926.41787	1463.71257	976.14414	732.35993	586.08940	28
11	1137.56879	569.28803	379.86111	285.14765	228.31958	P	2829.36510	1415.18619	943.79322	708.09673	566.67884	27
12	1234.62156	617.81442	412.21204	309.41085	247.73013	P	2732.31233	1366.65980	911.44229	683.83354	547.26829	26
13	1347.70563	674.35645	449.90673	337.68186	270.34695	L	2635.25956	1318.13342	879.09137	659.57035	527.85773	25
14	1404.72710	702.86719	468.91388	351.93723	281.75124	G	2522.17549	1261.59138	841.39668	631.29933	505.24092	24
15	1491.75913	746.38320	497.92456	373.69524	299.15765	S	2465.15402	1233.08065	822.38952	617.04396	493.83663	23
16	1620.80173	810.90450	540.93876	405.95589	324.96617	E	2378.12199	1189.56463	793.37885	595.28596	476.43022	22
17	1757.86064	879.43396	586.62506	440.22062	352.37795	H	2249.07939	1125.04333	750.36465	563.02531	450.62170	21
18	1871.90357	936.45542	624.63937	468.73135	375.18653	N	2112.02048	1056.51388	704.67834	528.76058	423.20992	20
19	1928.92504	964.96616	643.64653	482.98672	386.59083	G	1997.97755	999.49241	666.66403	500.24985	400.40133	19
20	2056.98362	1028.99545	686.33272	515.00136	412.20254	Q	1940.95608	970.98168	647.65688	485.99448	388.99704	18
21	2156.05204	1078.52966	719.35553	539.76847	432.01623	V	1812.89750	906.95239	604.97068	453.97983	363.38532	17
22	2227.08916	1114.04822	743.03457	557.52775	446.22365	A	1713.82908	857.41818	571.94788	429.21273	343.57164	16
23	2356.13176	1178.56952	786.04877	589.78840	472.03217	E	1642.79196	821.89962	548.26884	411.45345	329.36421	15
24	2471.15871	1236.08299	824.39109	618.54513	495.03756	D	1513.74936	757.37832	505.25464	379.19280	303.55569	14
25	2542.19583	1271.60155	848.07013	636.30441	509.24499	A	1398.72241	699.86484	466.91232	350.43606	280.55030	13
26	2641.26425	1321.13576	881.09293	661.07152	529.05867	V	1327.68529	664.34628	443.23328	332.67678	266.34288	12
27	2728.29628	1364.65178	910.10361	682.82953	546.46508	S	1228.61687	614.81207	410.21047	307.90968	246.52920	11
28	2884.39740	1442.70234	962.13732	721.85481	577.68530	R	1141.58484	571.29606	381.19980	286.15167	229.12279	10
29	2981.45017	1491.22872	994.48824	746.11800	597.09585	P	985.48372	493.24550	329.16609	247.12639	197.90257	9
30	3109.54514	1555.27621	1037.18656	778.14174	622.71485	K	888.43095	444.71911	296.81517	222.86320	178.49201	8
31	3224.57209	1612.78968	1075.52888	806.89848	645.72024	D	760.33598	380.67163	254.11684	190.83945	152.87302	7
32	3339.59904	1670.30316	1113.87120	835.65522	668.72563	D	645.30903	323.15815	215.77453	162.08272	129.86763	6
33	3426.63107	1713.81917	1142.88187	857.41322	686.13203	S	530.28208	265.64468	177.43221	133.32598	106.86224	5
34	3525.69949	1763.35338	1175.90468	882.18033	705.94572	V	443.25005	222.12866	148.42153	111.56797	89.45583	4
35	3622.75226	1811.87977	1208.25560	906.44352	725.35627	P	344.18163	172.59445	115.39873	86.80087	69.64215	3
36	3751.79486	1876.40107	1251.26980	938.70417	751.16479	E	247.12886	124.06807	83.04780	62.53767	50.23159	2
37						V	118.08626	59.54677	40.03360	30.27702	24.42307	1



Sequence: PKDDSVPEVRAARNSEPQ, Charge: +2, Monoisotopic m/z: 997.99738 Da (-0.82 mmu/-0.82 ppm), MH+: 1994.98747 Da, RT: 20.37 min,
Identified with: Sequest HT (v1.3); XCorr:0.77, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

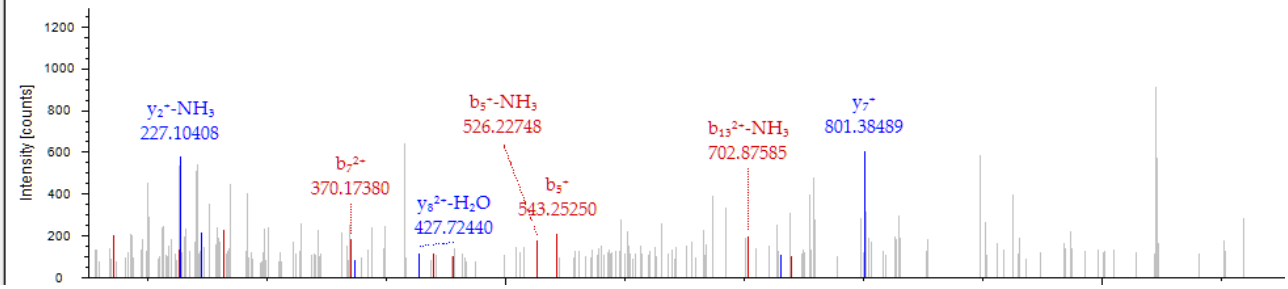
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	98.06005	49.53366	P			18
2	226.15502	113.58115	K	1897.93634	949.47181	17
3	341.18197	171.09462	D	1769.84137	885.42432	16
4	456.20892	228.60810	D	1654.81442	827.91085	15
5	543.24095	272.12411	S	1539.78747	770.39737	14
6	642.30937	321.65832	V	1452.75544	726.88136	13
7	739.36214	370.18471	P	1353.68702	677.34715	12
8	868.40474	434.70601	E	1256.63425	628.82076	11
9	967.47316	484.24022	V	1127.59165	564.29946	10
10	1123.57428	562.29078	R	1028.52323	514.76525	9
11	1194.61140	597.80934	A	872.42211	436.71469	8
12	1265.64852	633.32790	A	801.38499	401.19613	7
13	1421.74964	711.37846	R	730.34787	365.67757	6
14	1535.79257	768.39992	N	574.24675	287.62701	5
15	1622.82460	811.91594	S	460.20382	230.60555	4
16	1751.86720	876.43724	E	373.17179	187.08953	3
17	1848.91997	924.96362	P	244.12919	122.56823	2
18			Q	147.07642	74.04185	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #2642 RT: 20.37
FTMS, HCD@40.00, z=+2, Mono m/z=997.99738 Da, MH+=1994.98747 Da, Match Tol.=0.02 Da



Sequence: PAVPAGSQQGTPEEAAEALL, L20-Amidated (-0.98402 Da)
Charge: +2, Monoisotopic m/z: 967.99121 Da (-3.38 mmu/-3.49 ppm), MH+: 1934.97515 Da, RT: 35.60 min,
Identified with: Sequest HT (v1.3); XCorr:0.70, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

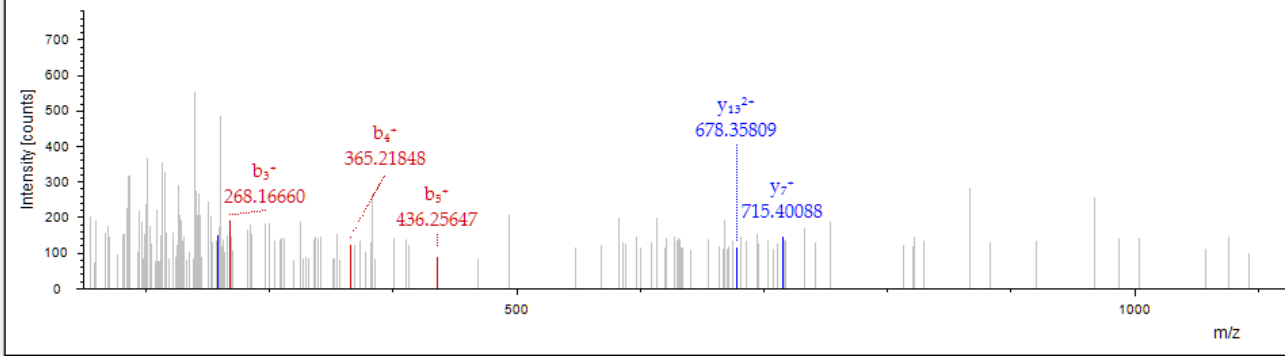
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	98.06005	49.53366	P			20
2	169.09717	85.05222	A	1837.92914	919.46821	19
3	268.16559	134.58643	V	1766.89202	883.94965	18
4	365.21836	183.11282	P	1667.82360	834.41544	17
5	436.25548	218.63138	A	1570.77083	785.88905	16
6	493.27695	247.14211	G	1499.73371	750.37049	15
7	580.30898	290.65813	S	1442.71224	721.85976	14
8	708.36756	354.68742	Q	1355.68021	678.34374	13
9	836.42614	418.71671	Q	1227.62163	614.31445	12
10	893.44761	447.22744	G	1099.56305	550.28516	11
11	994.49529	497.75128	T	1042.54158	521.77443	10
12	1091.54806	546.27767	P	941.49390	471.25059	9
13	1220.59066	610.79897	E	844.44113	422.72420	8
14	1349.63326	675.32027	E	715.39853	358.20290	7
15	1420.67038	710.83883	A	586.35593	293.68160	6
16	1491.70750	746.35739	A	515.31881	258.16304	5
17	1620.75010	810.87869	E	444.28169	222.64448	4
18	1691.78722	846.39725	A	315.23909	158.12318	3
19	1804.87129	902.93928	L	244.20197	122.60462	2
20			L-Amidated	131.11790	66.06259	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #4855 RT: 35.60
FTMS, HCD@40.00, z=+2, Mono m/z=967.99121 Da, MH+=1934.97515 Da, Match Tol.=0.02 Da



Sequence: QQETAAAETETRTHT, Q1-Gln->pyro-Glu (-17.02655 Da)
Charge: +2, Monoisotopic m/z: 828.88080 Da (+4.14 mmu/+5 ppm), MH+: 1656.75432 Da, RT: 14.41 min,
Identified with: Sequest HT (v1.3); XCorr:1.36, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

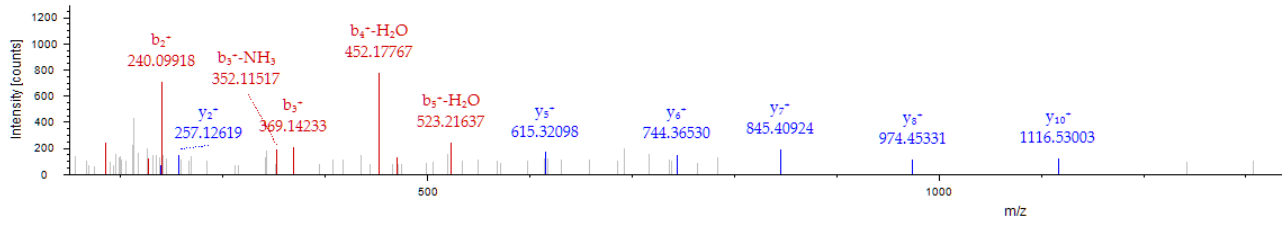
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	112.03931	56.52329	Q-Gln->pyr...			15
2	240.09789	120.55258	Q	1545.71401	773.36064	14
3	369.14049	185.07388	E	1417.65543	709.33135	13
4	470.18817	235.59772	T	1288.61283	644.81005	12
5	541.22529	271.11628	A	1187.56515	594.28621	11
6	612.26241	306.63484	A	1116.52803	558.76765	10
7	683.29953	342.15340	A	1045.49091	523.24909	9
8	812.34213	406.67470	E	974.45379	487.73053	8
9	913.38981	457.19854	T	845.41119	423.20923	7
10	1042.43241	521.71984	E	744.36351	372.68539	6
11	1143.48009	572.24368	T	615.32091	308.16409	5
12	1299.58121	650.29424	R	514.27323	257.64025	4
13	1400.62889	700.81808	T	358.17211	179.58969	3
14	1537.68780	769.34754	H	257.12443	129.06585	2
15			T	120.06552	60.53640	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_3.raw #1743 RT: 14.41
FTMS, HCD@40.00, z=+2, Mono m/z=828.88080 Da, MH+=1656.75432 Da, Match Tol.=0.02 Da



Sequence: QQETAAAETETRTHTLTRVNLESPGPERVW, Q1-Gln->pyro-Glu (-17.02655 Da)
Charge: +4, Monoisotopic m/z: 848.42828 Da (+5.97 mmu/+7.04 ppm), MH+: 3390.69130 Da, RT: 22.24 min,
Identified with: Sequest HT (v1.3); XCorr:5.55, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

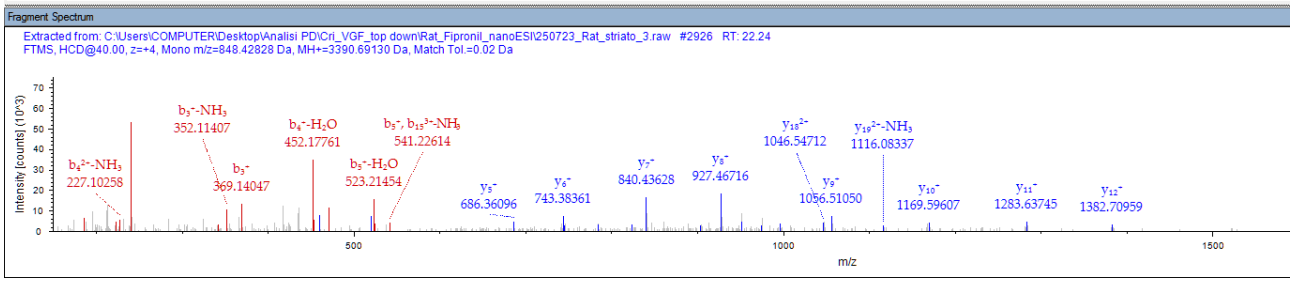
Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	#2
1	112.03931	56.52329	38.01795	28.76528	Q-Gln->pyr..					30
2	240.09789	120.55258	80.70415	60.77993	Q	3279.63540	1640.32134	1093.88332	820.66431	29
3	369.14049	185.07388	123.71835	93.04058	E	3151.57682	1576.29205	1051.19712	788.64966	28
4	470.18817	235.59772	157.40091	118.30250	T	3022.53422	1511.77075	1008.18292	756.38901	27
5	541.22529	271.11628	181.07995	136.06178	A	2921.48654	1461.24691	974.50036	731.12709	26
6	612.26241	306.63484	204.75899	153.82106	A	2850.44942	1425.72835	950.82132	713.36781	25
7	683.29953	342.15340	228.43803	171.58034	A	2779.41230	1390.20979	927.14228	695.60853	24
8	812.34213	406.67470	271.45223	203.84099	E	2708.37518	1354.69123	903.46324	677.84925	23
9	913.38981	457.19854	305.13479	229.10291	T	2579.33258	1290.16993	860.44904	645.58860	22
10	1042.43241	521.71984	348.14899	261.36356	E	2478.28490	1239.64609	826.76648	620.32668	21
11	1143.48009	572.24368	381.83155	286.62548	T	2349.24230	1175.12479	783.75228	588.06603	20
12	1299.58121	650.29424	433.86525	325.65076	R	2248.19462	1124.60095	750.06972	562.80411	19
13	1400.62889	700.81808	467.54781	350.91268	T	2092.09350	1046.55039	698.03602	523.77883	18
14	1537.68780	769.34754	513.23412	385.17741	H	1991.04582	996.02655	664.35346	498.51691	17
15	1638.73548	819.87138	546.91668	410.43933	T	1853.98691	927.49709	618.66715	464.25219	16
16	1751.81955	876.41341	584.61137	438.71034	L	1752.93923	876.97325	584.98459	438.99027	15
17	1852.86723	926.93725	618.29393	463.97226	T	1639.85516	820.43122	547.28990	410.71925	14
18	2008.96835	1004.98781	670.32763	502.99754	R	1538.80748	769.90738	513.60734	385.45733	13
19	2108.03677	1054.52202	703.35044	527.76465	V	1382.70636	691.85682	461.57364	346.43205	12
20	2222.07970	1111.54349	741.36475	556.27538	N	1283.63794	642.32261	428.55083	321.66494	11
21	2335.16377	1168.08552	779.05944	584.54640	L	1169.59501	585.30114	390.53652	293.15421	10
22	2464.20637	1232.60682	822.07364	616.80705	E	1056.51094	528.75911	352.84183	264.88319	9
23	2551.23840	1276.12284	851.08432	638.56506	S	927.46834	464.23781	309.82763	232.62254	8
24	2648.29117	1324.64922	883.43524	662.82825	P	840.43631	420.72179	280.81695	210.86454	7
25	2705.31264	1353.15996	902.44240	677.08362	G	743.38354	372.19541	248.46603	186.60134	6
26	2802.36541	1401.68634	934.79332	701.34681	P	686.36207	343.68467	229.45887	172.34598	5
27	2931.40801	1466.20764	977.80752	733.60746	E	589.30930	295.15829	197.10795	148.08278	4
28	3087.50913	1544.25820	1029.84123	772.63274	R	460.26670	230.63699	154.09375	115.82213	3
29	3186.57755	1593.79241	1062.86403	797.39984	V	304.16558	152.58643	102.06004	76.79685	2
30					W	205.09716	103.05222	69.03724	52.02975	1

Fragment Spectrum



Sequence: TAAAETETRTHTLTRVNLESPGPERVWRA, Charge: +4, Monoisotopic m/z: 813.17450 Da (+0.93 mmu/+1.14 ppm), MH+: 3249.67617 Da, RT: 19.08 min,
Identified with: Sequest HT (v1.3); XCorr:1.79, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

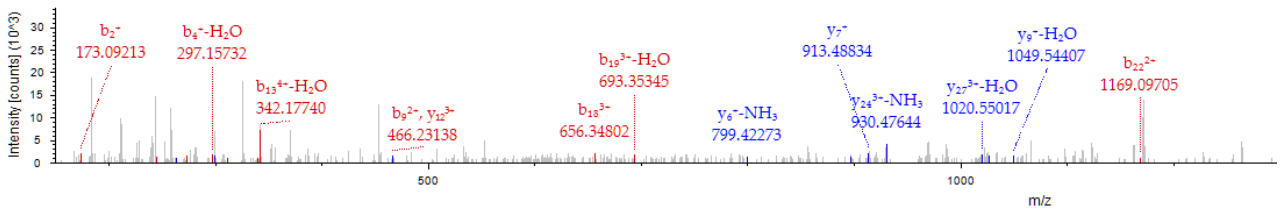
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	#2
1	102.05496	51.53112	34.68984	26.26920	T					29
2	173.09208	87.04968	58.36888	44.02848	A	3148.62478	1574.81603	1050.21311	787.91165	28
3	244.12920	122.56824	82.04792	61.78776	A	3077.58766	1539.29747	1026.53407	770.15237	27
4	315.16632	158.08680	105.72696	79.54704	A	3006.55054	1503.77891	1002.85503	752.39309	26
5	444.20892	222.60810	148.74116	111.80769	E	2935.51342	1468.26035	979.17599	734.63381	25
6	545.25660	273.13194	182.42372	137.06961	T	2806.47082	1403.73905	936.16179	702.37316	24
7	674.29920	337.65324	225.43792	169.33026	E	2705.42314	1353.21521	902.47923	677.11124	23
8	775.34688	388.17708	259.12048	194.59218	T	2576.38054	1288.69391	859.46503	644.85059	22
9	931.44800	466.22764	311.15418	233.61746	R	2475.33286	1238.17007	825.78247	619.58867	21
10	1032.49568	516.75148	344.83674	258.87938	T	2319.23174	1160.11951	773.74876	580.56339	20
11	1169.55459	585.28093	390.52305	293.14410	H	2218.18406	1109.59567	740.06620	555.30147	19
12	1270.60227	635.80477	424.20561	318.40602	T	2081.12515	1041.06621	694.37990	521.03675	18
13	1383.68634	692.34681	461.90030	346.67704	L	1980.07747	990.54237	660.69734	495.77483	17
14	1484.73402	742.87065	495.58286	371.93896	T	1866.99340	934.00034	623.00265	467.50381	16
15	1640.83514	820.92121	547.61656	410.96424	R	1765.94572	883.47650	589.32009	442.24189	15
16	1739.90356	870.45542	580.63937	435.73135	V	1609.84460	805.42594	537.28638	403.21661	14
17	1853.94649	927.47688	618.65368	464.24208	N	1510.77618	755.89173	504.26358	378.44950	13
18	1967.03056	984.01892	656.34837	492.51310	L	1396.73325	698.87026	466.24927	349.93877	12
19	2096.07316	1048.54022	699.36257	524.77375	E	1283.64918	642.32823	428.55458	321.66775	11
20	2183.10519	1092.05623	728.37325	546.53175	S	1154.60658	577.80693	385.54038	289.40710	10
21	2280.15796	1140.58262	760.72417	570.79495	P	1067.57455	534.29091	356.52970	267.64910	9
22	2337.17943	1169.09335	779.73133	585.05031	G	970.52178	485.76453	324.17878	243.38590	8
23	2434.23220	1217.61974	812.08225	609.31351	P	913.50031	457.25379	305.17162	229.13054	7
24	2563.27480	1282.14104	855.09645	641.57416	E	816.44754	408.72741	272.82070	204.86734	6
25	2719.37592	1360.19160	907.13016	680.59944	R	687.40494	344.20611	229.80650	172.60669	5
26	2818.44434	1409.72581	940.15296	705.36654	V	531.30382	266.15555	177.77279	133.58141	4
27	3004.52366	1502.76547	1002.17940	751.88637	W	432.23540	216.62134	144.74998	108.81431	3
28	3160.62478	1580.81603	1054.21311	790.91165	R	246.15608	123.58168	82.72354	62.29448	2
29					A	90.05496	45.53112	30.68984	23.26920	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #2463 RT: 19.08
FTMS, HCD@40.00, z=+4, Mono m/z=813.17450 Da, MH+=3249.67617 Da, Match Tol.=0.02 Da



Sequence: MSENVPLPETHQFGEGVSSPKTHLGETLTPLSKAYQSLSAPFPKV, Charge: +5, Monoisotopic m/z: 968.09961 Da (+2.69 mmu/+2.78 ppm), MH+: 4836.46894 Da, RT: 31.92 min,
Identified with: Sequest HT (v1.3); XCorr:3.25, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

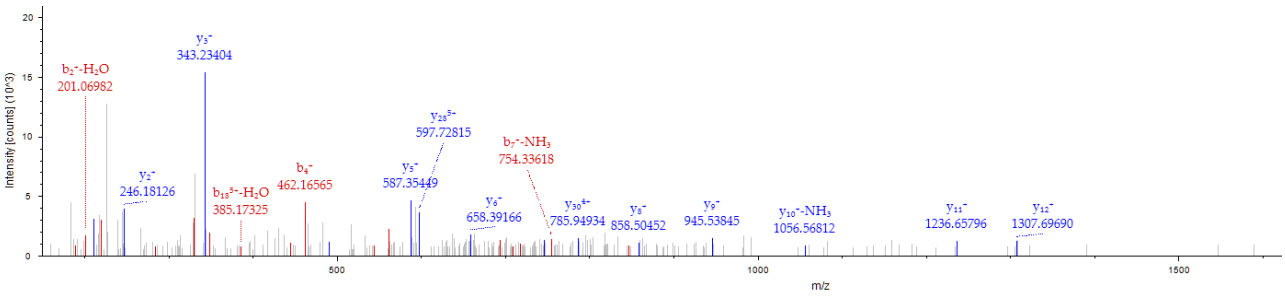
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	b^{4+}	b^{5+}	Seq.	y^+	y^{2+}	y^{3+}	y^{4+}	y^{5+}	#2
1	132.04778	66.52753	44.68744	33.76740	27.21538	M						45
2	219.07981	110.04354	73.69812	55.52541	44.62178	S	4705.41500	2353.21114	1569.14318	1177.10921	941.88882	44
3	348.12241	174.56484	116.71232	87.78606	70.43030	E	4618.38297	2309.69512	1540.13251	1155.35120	924.48242	43
4	462.16534	231.58631	154.72663	116.29679	93.23889	N	4489.34037	2245.17382	1497.11831	1123.09055	898.67390	42
5	561.23376	281.12052	187.74944	141.06390	113.05257	V	4375.29744	2188.15236	1459.10400	1094.57982	875.86531	41
6	658.28653	329.64690	220.10036	165.32709	132.46313	P	4276.22902	2138.61815	1426.08119	1069.81271	856.05163	40
7	771.37060	386.18894	257.79505	193.59811	155.07994	L	4179.17625	2090.09176	1393.73027	1045.54952	836.64107	39
8	868.42337	434.71532	290.14597	217.86130	174.49049	P	4066.09218	2033.54973	1356.03558	1017.27850	814.02426	38
9	997.46597	499.23662	333.16017	250.12195	200.29901	E	3969.03941	1985.02334	1323.68465	993.01531	794.61370	37
10	1098.51365	549.76046	366.84273	275.38387	220.50855	T	3839.99681	1920.50204	1280.67045	960.75466	768.80518	36
11	1235.57256	618.28992	412.52904	309.64860	247.92033	H	3738.94913	1869.97820	1246.98789	935.49274	748.59565	35
12	1363.63114	682.31921	455.21523	341.66324	273.53205	Q	3601.89022	1801.44875	1201.30159	901.22801	721.18387	34
13	1510.69956	755.85342	504.23804	378.43035	302.94573	F	3473.83164	1737.41946	1158.61540	869.21337	695.57215	33
14	1567.72103	784.36415	523.24519	392.68571	314.35003	G	3326.76322	1663.88525	1109.59259	832.44626	666.15847	32
15	1696.76363	848.88545	566.25939	424.94636	340.15855	E	3269.74175	1635.37451	1090.58543	818.19090	654.75417	31
16	1753.78510	877.39619	585.26655	439.20173	351.56284	G	3140.69915	1570.85321	1047.57123	785.93025	628.94565	30
17	1852.85352	926.93040	618.28936	463.96884	371.37652	V	3083.67768	1542.34248	1028.56408	771.67488	617.54136	29
18	1939.88555	970.44641	647.30003	485.72684	388.78293	S	2984.60926	1492.80827	995.54127	746.90777	597.72767	28
19	2026.91758	1013.96243	676.31071	507.48485	406.18934	S	2897.57723	1449.29225	966.53059	725.14977	580.32127	27
20	2123.97035	1062.48881	708.66163	531.74804	425.59989	P	2810.54520	1405.77624	937.51992	703.39176	562.91486	26
21	2252.06532	1126.53630	751.35996	563.77179	451.21888	K	2713.49243	1357.24985	905.16899	679.12857	543.50431	25
22	2353.11300	1177.06014	785.04252	589.03371	471.42842	T	2585.39746	1293.20237	862.47067	647.10482	517.88531	24
23	2490.17191	1245.58959	830.72882	623.29843	498.84020	H	2484.34978	1242.67853	828.78811	621.84290	497.67578	23
24	2603.25598	1302.13163	868.42351	651.56945	521.45702	L	2347.29087	1174.14907	783.10181	587.57818	470.26400	22
25	2660.27745	1330.64236	887.43067	665.82482	532.86131	G	2234.20680	1117.60704	745.40712	559.30716	447.64718	21
26	2789.32005	1395.16366	930.44487	698.08547	558.66983	E	2177.18533	1089.09630	726.39996	545.05179	436.24289	20
27	2890.36773	1445.68750	964.12743	723.34739	578.87937	T	2048.14273	1024.57500	683.38576	512.79114	410.43437	19
28	3003.45180	1502.22954	1001.82212	751.61841	601.49618	L	1947.09505	974.05116	649.70320	487.52922	390.22483	18
29	3104.49948	1552.75338	1035.50468	776.88033	621.70572	T	1834.01098	917.50913	612.00851	459.25820	367.60802	17
30	3201.55225	1601.27976	1067.85560	801.14352	641.11627	P	1732.96330	866.98529	578.32595	433.99628	347.39848	16
31	3314.63632	1657.82180	1105.55029	829.41454	663.73308	L	1635.91053	818.45890	545.97503	409.73309	327.98793	15
32	3401.66835	1701.33781	1134.56097	851.17254	681.13949	S	1522.82646	761.91687	508.28034	381.46207	305.37111	14
33	3529.76332	1765.38530	1177.25929	883.19629	706.75848	K	1435.79443	718.40085	479.26966	359.70407	287.96471	13
34	3600.80044	1800.90386	1200.93833	900.95557	720.96591	A	1307.69946	654.35337	436.57134	327.68032	262.34571	12
35	3763.86376	1882.43552	1255.29277	941.72140	753.57857	Y	1236.66234	618.83481	412.89230	309.92104	248.13829	11
36	3891.92234	1946.46481	1297.97896	973.73604	779.19029	Q	1073.59902	537.30315	358.53786	269.15521	215.52563	10
37	3978.95437	1989.98082	1326.98964	995.49405	796.59669	S	945.54044	473.27386	315.85166	237.14057	189.91391	9
38	4092.03844	2046.52286	1364.68433	1023.76507	819.21351	L	858.50841	429.75784	286.84099	215.38256	172.50750	8
39	4179.07047	2090.03887	1393.69501	1045.52307	836.61991	S	745.42434	373.21581	249.14630	187.11154	149.89069	7
40	4250.10759	2125.55743	1417.37405	1063.28235	850.82734	A	658.39231	329.69979	220.13562	165.35354	132.48428	6
41	4347.16036	2174.08382	1449.72497	1087.54555	870.23789	P	587.35519	294.18123	196.45658	147.59426	118.27686	5
42	4494.22878	2247.61803	1498.74778	1124.31265	899.65158	F	490.30242	245.65485	164.10566	123.33106	98.86631	4
43	4591.28155	2296.14441	1531.09870	1148.57584	919.06213	P	343.23400	172.12064	115.08285	86.56396	69.45262	3
44	4719.37652	2360.19190	1573.79702	1180.59959	944.68112	K	246.18123	123.59425	82.73193	62.30077	50.04207	2
45						V	118.08626	59.54677	40.03360	30.27702	24.42307	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #4290 RT: 31.92
FTMS, HCD@40.00, z=+5, Mono m/z=968.09961 Da, MH+=4836.46894 Da, Match Tol.=0.02 Da



Sequence: QVEAGRRQAEATRQAAAQEERLADLASDLL, Q1-Gln->pyro-Glu (-17.02655 Da)
Charge: +4, Monoisotopic m/z: 813.17450 Da (+4.74 mmu/+5.83 ppm), MH+: 3249.67617 Da, RT: 19.08 min,
Identified with: Sequest HT (v1.3); XCorr:2.00, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

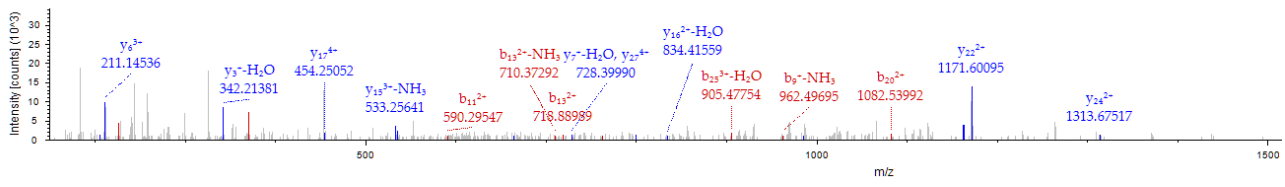
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	b4+	Seq.	y+	y2+	y3+	y4+	#2
1	112.03931	56.52329	38.01795	28.76528	Q-Gln->pyr...					30
2	211.10773	106.05750	71.04076	53.53239	V	3138.62520	1569.81624	1046.87992	785.41176	29
3	340.15033	170.57880	114.05496	85.79304	E	3039.55678	1520.28203	1013.85711	760.64465	28
4	411.18745	206.09736	137.73400	103.55232	A	2910.51418	1455.76073	970.84291	728.38400	27
5	468.20892	234.60810	156.74116	117.80769	G	2839.47706	1420.24217	947.16387	710.62472	26
6	624.31004	312.65866	208.77486	156.83297	R	2782.45559	1391.73143	928.15671	696.36936	25
7	780.41116	390.70922	260.80857	195.85825	R	2626.35447	1313.68087	876.12301	657.34408	24
8	908.46974	454.73851	303.49476	227.87289	Q	2470.25335	1235.63031	824.08930	618.31880	23
9	979.50686	490.25707	327.17380	245.63217	A	2342.19477	1171.60102	781.40311	586.30415	22
10	1108.54946	554.77837	370.18800	277.89282	E	2271.15765	1136.08246	757.72407	568.54487	21
11	1179.58658	590.29693	393.86704	295.65210	A	2142.11505	1071.56116	714.70987	536.28422	20
12	1280.63426	640.82077	427.54960	320.91402	T	2071.07793	1036.04260	691.03083	518.52494	19
13	1436.73538	718.87133	479.58331	359.93930	R	1970.03025	985.51876	657.34827	493.26302	18
14	1564.79396	782.90062	522.26950	391.95395	Q	1813.92913	907.46820	605.31456	454.23774	17
15	1635.83108	818.41918	545.94854	409.71323	A	1685.87055	843.43891	562.62837	422.22310	16
16	1706.86820	853.93774	569.62758	427.47251	A	1614.83343	807.92035	538.94933	404.46382	15
17	1777.90532	889.45630	593.30662	445.23179	A	1543.79631	772.40179	515.27029	386.70454	14
18	1905.96390	953.48559	635.99282	477.24643	Q	1472.75919	736.88323	491.59125	368.94526	13
19	2035.00650	1018.00689	679.00702	509.50708	E	1344.70061	672.85394	448.90505	336.93061	12
20	2164.04910	1082.52819	722.02122	541.76773	E	1215.65801	608.33264	405.89085	304.66996	11
21	2320.15022	1160.57875	774.05492	580.79301	R	1086.61541	543.81134	362.87665	272.40931	10
22	2433.23429	1217.12078	811.74961	609.06403	L	930.51429	465.76078	310.84295	233.38403	9
23	2504.27141	1252.63934	835.42865	626.82331	A	817.43022	409.21875	273.14826	205.11301	8
24	2619.29836	1310.15282	873.77097	655.58005	D	746.39310	373.70019	249.46922	187.35373	7
25	2732.38243	1366.69485	911.46566	683.85106	L	631.36615	316.18671	211.12690	158.59700	6
26	2803.41955	1402.21341	935.14470	701.61034	A	518.28208	259.64468	173.43221	130.32598	5
27	2890.45158	1445.72943	964.15538	723.36835	S	447.24496	224.12612	149.75317	112.56670	4
28	3005.47853	1503.24290	1002.49769	752.12509	D	360.21293	180.61010	120.74249	90.80869	3
29	3118.56260	1559.78494	1040.19238	780.39611	L	245.18598	123.09663	82.40018	62.05195	2
30					L	132.10191	66.55459	44.70549	33.78094	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #2463 RT: 19.08
FTMS, HCD@40.00, z=+4, Mono m/z=813.17450 Da, MH+=3249.67617 Da, Match Tol.=0.02 Da



Sequence: QVEAGRRQAEATRQAAAQEERLADLASDLLLQYLLQGGARQRDLGGRGLQETQQER, R56-Amidated (-0.98402 Da)
Charge: +6, Monoisotopic m/z: 1037.03992 Da (-6.77 mmu/-6.53 ppm), MH+: 6217.20312 Da, RT: 40.05 min,
aults tified with: Sequest HT (v1.3); XCorr:2.23, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

Value Type: Theo. Mass [Da]

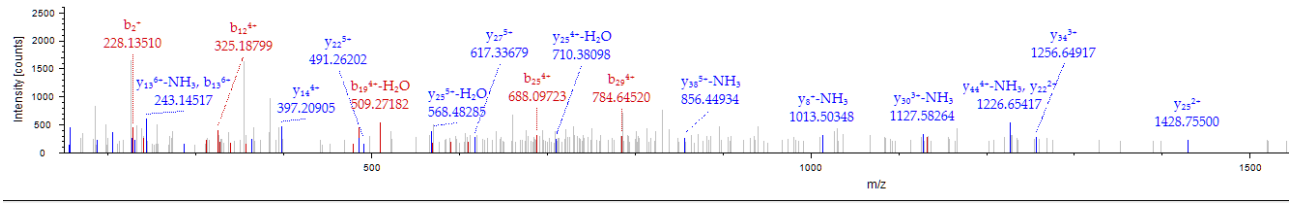
Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	b4+	b5+	b6+	Seq.	y+	y2+	y3+	y4+	y5+	y6+	#2
1	129.06586	65.03657	43.69347	33.02192	26.61899	22.35037	Q							56
2	228.13428	114.57078	76.71628	57.78903	46.43268	38.86178	V	6089.18512	3045.09620	2030.39989	1523.05174	1218.64284	1015.70358	55
3	357.17688	179.09208	119.73048	90.04968	72.24120	60.36888	E	5990.11670	2995.56199	1997.37708	1498.28463	1198.82916	999.19218	54
4	428.21400	214.61064	143.40952	107.80896	86.44862	72.20840	A	5861.07410	2931.04069	1954.36288	1466.02398	1173.02064	977.68508	53
5	485.23547	243.12137	162.41667	122.06432	97.85291	81.71197	G	5790.03698	2895.52213	1930.68384	1448.26470	1158.81322	965.84556	52
6	641.33659	321.17193	214.45038	161.08960	129.07314	107.72883	R	5733.01551	2867.01139	1911.67669	1434.00933	1147.40892	956.34198	51
7	797.43771	399.22249	266.48409	200.11488	160.29336	133.74568	R	5576.91439	2788.96083	1859.64298	1394.98405	1116.18870	930.32513	50
8	925.49629	463.25178	309.17028	232.12953	185.90508	155.08878	Q	5420.81327	2710.91027	1807.60927	1355.95877	1084.96847	904.30827	49
9	996.53341	498.77034	332.84932	249.88881	200.11250	166.92830	A	5292.75469	2646.88098	1764.92308	1323.94413	1059.35676	882.96518	48
10	1125.57601	563.29164	375.86352	282.14946	225.92102	188.43540	E	5221.71757	2611.36242	1741.24404	1306.18485	1045.14933	871.12566	47
11	1196.61313	598.81020	399.54256	299.90874	240.12845	200.27492	A	5092.67497	2546.84112	1698.22984	1273.92420	1019.34081	849.61856	46
12	1297.66081	649.33404	433.22512	325.17066	260.33798	217.11620	T	5021.63785	2511.32256	1674.55080	1256.16492	1005.13339	837.77904	45
13	1453.76193	727.38460	485.25883	364.19594	291.55821	243.13305	R	4920.59017	2460.79872	1640.86824	1230.90300	984.92385	820.93776	44
14	1581.82051	791.41389	527.94502	396.21058	317.16992	264.47615	Q	4764.48905	2382.74816	1588.83453	1191.87772	953.70363	794.92090	43
15	1652.85763	826.93245	551.62406	413.96986	331.37735	276.31567	A	4636.43047	2318.71887	1546.14834	1159.86307	928.09191	773.57781	42
16	1723.89475	862.45101	575.30310	431.72914	345.58477	288.15519	A	4565.39335	2283.20031	1522.46930	1142.10379	913.88449	761.73829	41
17	1794.93187	897.96957	598.98214	449.48842	359.79219	299.99471	A	4494.35623	2247.68175	1498.79026	1124.34451	899.67707	749.89877	40
18	1922.99045	961.99886	641.66833	481.50307	385.40391	321.33780	Q	4423.31911	2212.16319	1475.11122	1106.58523	885.46964	738.05925	39
19	2052.03305	1026.52016	684.68253	513.76372	411.21243	342.84490	E	4295.26053	2148.13390	1432.42503	1074.57059	859.85793	716.71615	38
20	2181.07565	1091.04146	727.69673	546.02437	437.02095	364.35200	E	4166.21793	2083.61260	1389.41083	1042.30994	834.04941	695.20905	37
21	2337.17677	1169.09202	779.73044	585.04965	468.24117	390.36886	R	4037.17533	2019.09130	1346.39663	1010.04929	808.24089	673.70195	36
22	2450.26084	1225.63406	817.42513	613.32067	490.85799	409.21620	L	3881.07421	1941.04074	1294.36292	971.02401	777.02066	647.68510	35
23	2521.29796	1261.15262	841.10417	631.07995	505.06541	421.05572	A	3767.99014	1884.49871	1256.66823	942.75299	754.40385	628.83775	34
24	2636.32491	1318.66609	879.44649	659.83668	528.07080	440.22688	D	3696.95302	1848.98015	1232.98919	924.99371	740.19642	616.99823	33
25	2749.40898	1375.20813	917.14118	688.10770	550.68762	459.07423	L	3581.92607	1791.46667	1194.64687	896.23697	717.19103	597.82707	32

26	2820.44610	1410.72669	940.82022	705.86698	564.89504	470.91375	A	3468.84200	1734.92464	1156.95218	867.96596	694.57422	578.97973	31
27	2907.47813	1454.24270	969.83089	727.62499	582.30145	485.41908	S	3397.80488	1699.40608	1133.27314	850.20668	680.36680	567.14021	30
28	3022.50508	1511.75618	1008.17321	756.38173	605.30684	504.59024	D	3310.77285	1655.89006	1104.26247	828.44867	662.96039	552.63487	29
29	3135.58915	1568.29821	1045.86790	784.65274	627.92365	523.43759	L	3195.74590	1598.37659	1065.92015	799.69193	639.95500	533.46371	28
30	3248.67322	1624.84025	1083.56259	812.92376	650.54046	542.28493	L	3082.66183	1541.83455	1028.22546	771.42091	617.33819	514.61637	27
31	3361.75729	1681.38228	1121.25728	841.19478	673.15728	561.13228	L	2969.57776	1485.29252	990.53077	743.14990	594.72137	495.76902	26
32	3489.81587	1745.41157	1163.94347	873.20942	698.76899	582.47537	Q	2856.49369	1428.75048	952.83608	714.87888	572.10456	476.92168	25
33	3652.87919	1826.94323	1218.29791	913.97525	731.38166	609.65259	Y	2728.43511	1364.72119	910.14989	682.86423	546.49284	455.57858	24
34	3765.96326	1883.48527	1255.99260	942.24627	753.99847	628.49994	L	2565.37179	1283.18953	855.79545	642.09840	513.88018	428.40136	23
35	3879.04733	1940.02730	1293.68729	970.51729	776.61529	647.34728	L	2452.28772	1226.64750	818.10076	613.82739	491.26336	409.55402	22
36	4007.10591	2004.05659	1336.37349	1002.53193	802.22700	668.69038	Q	2339.20365	1170.10546	780.40607	585.55637	468.64655	390.70667	21
37	4064.12738	2032.56733	1355.38064	1016.78730	813.63130	678.19396	G	2211.14507	1106.07617	737.71987	553.54172	443.03483	369.36357	20
38	4121.14885	2061.07806	1374.38780	1031.04267	825.03559	687.69754	G	2154.12360	1077.56544	718.71272	539.28636	431.63054	359.86000	19
39	4192.18597	2096.59662	1398.06684	1048.80195	839.24301	699.53706	A	2097.10213	1049.05470	699.70556	525.03099	420.22625	350.35642	18
40	4348.28709	2174.64718	1450.10055	1087.82723	870.46324	725.55391	R	2026.06501	1013.53614	676.02652	507.27171	406.01882	338.51690	17
41	4476.34567	2238.67647	1492.78674	1119.84187	896.07495	746.89701	Q	1869.96389	935.48558	623.99281	468.24643	374.79860	312.50004	16
42	4632.44679	2316.72703	1544.82045	1158.86715	927.29518	772.91386	R	1741.90531	871.45629	581.30662	436.23178	349.18688	291.15695	15
43	4747.47374	2374.24051	1583.16276	1187.62389	950.30057	792.08502	D	1585.80419	793.40573	529.27291	397.20650	317.96666	265.14009	14
44	4860.55781	2430.78254	1620.85745	1215.89491	972.91738	810.93236	L	1470.77724	735.89226	490.93060	368.44977	294.96127	245.96894	13
45	4917.57928	2459.29328	1639.86461	1230.15028	984.32168	820.43594	G	1357.69317	679.35022	453.23591	340.17875	272.34445	227.12159	12
46	4974.60075	2487.80401	1658.87177	1244.40564	995.72597	829.93952	G	1300.67170	650.83949	434.22875	325.92338	260.94016	217.61801	11
47	5130.70187	2565.85457	1710.90547	1283.43092	1026.94619	855.95637	R	1243.65023	622.32875	415.22159	311.66801	249.53587	208.11443	10
48	5187.72334	2594.36531	1729.91263	1297.68629	1038.35049	865.45995	G	1087.54911	544.27819	363.18789	272.64273	218.31564	182.09758	9
49	5300.80741	2650.90734	1767.60732	1325.95731	1060.96730	884.30730	L	1030.52764	515.76746	344.18073	258.38737	206.91135	172.59400	8
50	5428.86599	2714.93663	1810.29351	1357.97195	1086.57902	905.65039	Q	917.44357	459.22542	306.48604	230.11635	184.29453	153.74666	7
51	5557.90859	2779.45793	1853.30771	1390.23260	1112.38754	927.15749	E	789.38499	395.19613	263.79985	198.10170	158.68282	132.40356	6
52	5658.95627	2829.98177	1886.99027	1415.49452	1132.59707	943.99877	T	660.34239	330.67483	220.78565	165.84105	132.87430	110.89646	5
53	5787.01485	2894.01106	1929.67647	1447.50917	1158.20879	965.34187	Q	559.29471	280.15099	187.10309	140.57913	112.66476	94.05518	4
54	5915.07343	2958.04035	1972.36266	1479.52381	1183.82051	986.68497	Q	431.23613	216.12170	144.41689	108.56449	87.05305	72.71208	3
55	6044.11603	3022.56165	2015.37686	1511.78446	1209.62903	1008.19207	E	303.17755	152.09241	101.73070	76.54984	61.44133	51.36899	2
56							R-Amidated	174.13495	87.57111	58.71650	44.28919	35.63281	29.86189	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_FipronIl_nanoESI\250723_Rat_striato_3.raw #5575 RT: 40.05
FTMS, HCD@40.00, z=+6, Mono m/z=1037.03992 Da, MH+=6217.20312 Da, Match Tol.=0.02 Da



Sequence: GLQETQQERENEREEEAEQE, Charge: +3, Monoisotopic m/z: 821.02997 Da (+1.43 mmu/+1.74 ppm), MH+: 2461.07535 Da, RT: 13.38 min,
Identified with: Sequest HT (v1.3); XCorr:3.17, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

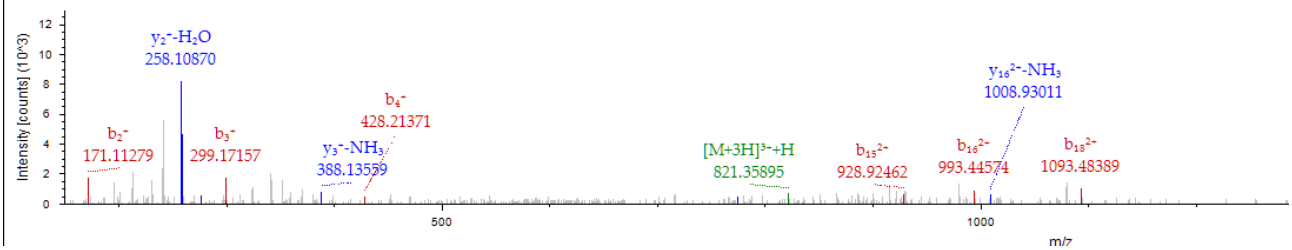
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	Seq.	y^+	y^{2+}	y^{3+}	#2
1	58.02875	29.51801	20.01443	G				20
2	171.11282	86.06005	57.70912	L	2404.04960	1202.52844	802.02138	19
3	299.17140	150.08934	100.39532	Q	2290.96553	1145.98640	764.32669	18
4	428.21400	214.61064	143.40952	E	2162.90695	1081.95711	721.64050	17
5	529.26168	265.13448	177.09208	T	2033.86435	1017.43581	678.62630	16
6	657.32026	329.16377	219.77827	Q	1932.81667	966.91197	644.94374	15
7	785.37884	393.19306	262.46446	Q	1804.75809	902.88268	602.25755	14
8	914.42144	457.71436	305.47866	E	1676.69951	838.85339	559.57135	13
9	1070.52256	535.76492	357.51237	R	1547.65691	774.33209	516.55715	12
10	1199.56516	600.28622	400.52657	E	1391.55579	696.28153	464.52345	11
11	1313.60809	657.30768	438.54088	N	1262.51319	631.76023	421.50925	10
12	1442.65069	721.82898	481.55508	E	1148.47026	574.73877	383.49494	9
13	1598.75181	799.87954	533.58879	R	1019.42766	510.21747	340.48074	8
14	1727.79441	864.40084	576.60299	E	863.32654	432.16691	288.44703	7
15	1856.83701	928.92214	619.61719	E	734.28394	367.64561	245.43283	6
16	1985.87961	993.44344	662.63139	E	605.24134	303.12431	202.41863	5
17	2056.91673	1028.96200	686.31043	A	476.19874	238.60301	159.40443	4
18	2185.95933	1093.48330	729.32463	E	405.16162	203.08445	135.72539	3
19	2314.01791	1157.51259	772.01082	Q	276.11902	138.56315	92.71119	2
20				E	148.06044	74.53386	50.02500	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_2.raw #1595 RT: 13.38
FTMS, HCD@40.00, z=+3, Mono m/z=821.02997 Da, MH+=2461.07535 Da, Match Tol.=0.02 Da



Sequence: TIDSLIELSTKLHLPADDVVSIIEEVEE, E28-Amidated (-0.98402 Da)
Charge: +4, Monoisotopic m/z: 777.42157 Da (+5.94 mmu/+7.64 ppm), MH+: 3106.66445 Da, RT: 23.65 min,
Defaults fied with: Sequest HT (v1.3); XCorr:1.98, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

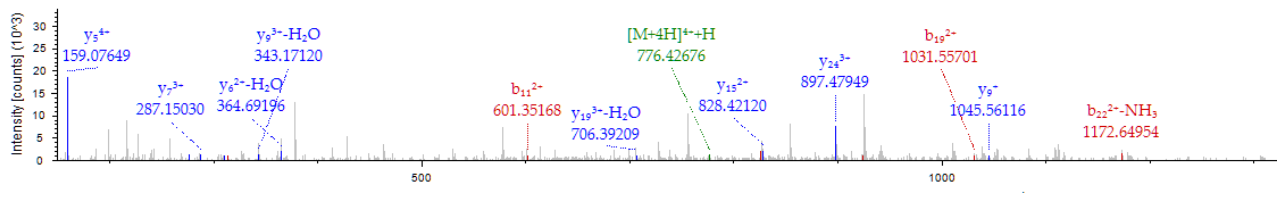
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	b4+	Seq.	y+	y2+	y3+	y4+	#2
1	102.05496	51.53112	34.68984	26.26920	T					28
2	215.13903	108.07315	72.38453	54.54021	I	3005.59304	1503.30016	1002.53586	752.15372	27
3	330.16598	165.58663	110.72684	83.29695	D	2892.50897	1446.75812	964.84117	723.88270	26
4	417.19801	209.10264	139.73752	105.05496	S	2777.48202	1389.24465	926.49886	695.12596	25
5	530.28208	265.64468	177.43221	133.32598	L	2690.44999	1345.72863	897.48818	673.36795	24
6	643.36615	322.18671	215.12690	161.59699	I	2577.36592	1289.18660	859.79349	645.09694	23
7	772.40875	386.70801	258.14110	193.85764	E	2464.28185	1232.64456	822.09880	616.82592	22
8	885.49282	443.25005	295.83579	222.12866	L	2335.23925	1168.12326	779.08460	584.56527	21
9	972.52485	486.76606	324.84647	243.88667	S	2222.15518	1111.58123	741.38991	556.29425	20
10	1073.57253	537.28990	358.52903	269.14859	T	2135.12315	1068.06521	712.37923	534.53624	19
11	1201.66750	601.33739	401.22735	301.17233	K	2034.07547	1017.54137	678.69667	509.27432	18
12	1314.75157	657.87942	438.92204	329.44335	L	1905.98050	953.49389	635.99835	477.25058	17
13	1451.81048	726.40888	484.60834	363.70808	H	1792.89643	896.95185	598.30366	448.97956	16
14	1564.89455	782.95091	522.30303	391.97909	L	1655.83752	828.42240	552.61736	414.71484	15
15	1661.94732	831.47730	554.65396	416.24229	P	1542.75345	771.88036	514.92267	386.44382	14
16	1732.98444	866.99586	578.33300	434.00157	A	1445.70068	723.35398	482.57174	362.18063	13
17	1848.01139	924.50933	616.67531	462.75830	D	1374.66356	687.83542	458.89270	344.42135	12
18	1963.03834	982.02281	655.01763	491.51504	D	1259.63661	630.32194	420.55039	315.66461	11
19	2062.10676	1031.55702	688.04044	516.28215	V	1144.60966	572.80847	382.20807	286.90787	10
20	2161.17518	1081.09123	721.06324	541.04925	V	1045.54124	523.27426	349.18526	262.14077	9
21	2248.20721	1124.60724	750.07392	562.80726	S	946.47282	473.74005	316.16246	237.37366	8
22	2361.29128	1181.14928	787.76861	591.07828	I	859.44079	430.22403	287.15178	215.61565	7
23	2474.37535	1237.69131	825.46330	619.34929	I	746.35672	373.68200	249.45709	187.34464	6
24	2603.41795	1302.21261	868.47750	651.60994	E	633.27265	317.13996	211.76240	159.07362	5
25	2732.46055	1366.73391	911.49170	683.87059	E	504.23005	252.61866	168.74820	126.81297	4
26	2831.52897	1416.26812	944.51451	708.63770	V	375.18745	188.09736	125.73400	94.55232	3
27	2960.57157	1480.78942	987.52871	740.89835	E	276.11903	138.56315	92.71119	69.78521	2
28					E-Amidated	147.07643	74.04185	49.69699	37.52456	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #3140 RT: 23.65
FTMS, HCD@40.00, z=+4, Mono m/z=777.42157 Da, MH+=3106.66445 Da, Match Tol.=0.02 Da



Peptide Summary

Sequence: KKNAPPEPVPPPRAAPAPTHV, Charge: +4, Monoisotopic m/z: 543.55914 Da (+1.56 mmu/+2.87 ppm), MH+: 2171.21474 Da, RT: 14.20 min,
Identified with: Sequest HT (v1.3); XCorr:3.78, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

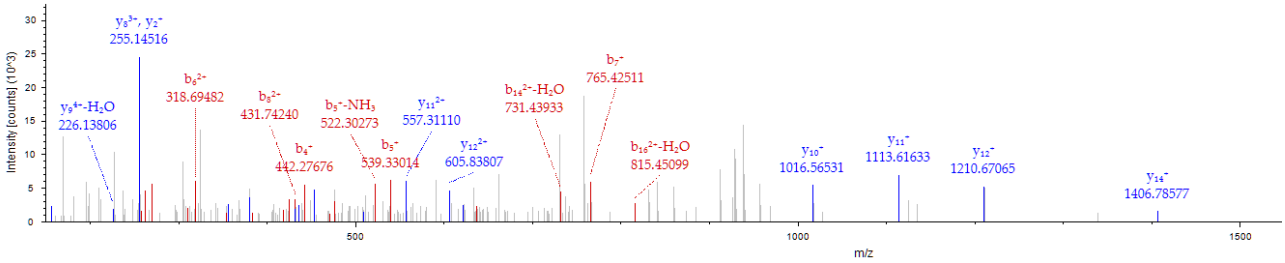
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b+	b2+	b3+	b4+	Seq.	y+	y2+	y3+	y4+	#2
1	129.10225	65.05476	43.70560	33.03102	K					21
2	257.19722	129.10225	86.40392	65.05476	K	2043.11353	1022.06040	681.70936	511.53384	20
3	371.24015	186.12371	124.41823	93.56549	N	1915.01856	958.01292	639.01104	479.51010	19
4	442.27727	221.64227	148.09727	111.32477	A	1800.97563	900.99145	600.99673	450.99937	18
5	539.33004	270.16866	180.44820	135.58797	P	1729.93851	865.47289	577.31769	433.24009	17
6	636.38281	318.69504	212.79912	159.85116	P	1632.88574	816.94651	544.96676	408.97689	16
7	765.42541	383.21634	255.81332	192.11181	E	1535.83297	768.42012	512.61584	384.71370	15
8	862.47818	431.74273	288.16424	216.37500	P	1406.79037	703.89882	469.60164	352.45305	14
9	961.54660	481.27694	321.18705	241.14211	V	1309.73760	655.37244	437.25072	328.18986	13
10	1058.59937	529.80332	353.53797	265.40530	P	1210.66918	605.83823	404.22791	303.42275	12
11	1155.65214	578.32971	385.88890	289.66849	P	1113.61641	557.31184	371.87699	279.15956	11
12	1252.70491	626.85609	418.23982	313.93168	P	1016.56364	508.78546	339.52606	254.89637	10
13	1408.80603	704.90665	470.27353	352.95696	R	919.51087	460.25907	307.17514	230.63318	9
14	1479.84315	740.42521	493.95257	370.71624	A	763.40975	382.20851	255.14143	191.60790	8
15	1550.88027	775.94377	517.63161	388.47552	A	692.37263	346.68995	231.46239	173.84862	7
16	1647.93304	824.47016	549.98253	412.73872	P	621.33551	311.17139	207.78335	156.08934	6
17	1718.97016	859.98872	573.66157	430.49800	A	524.28274	262.64501	175.43243	131.82614	5
18	1816.02293	908.51510	606.01249	454.76119	P	453.24562	227.12645	151.75339	114.06686	4
19	1917.07061	959.03894	639.69505	480.02311	T	356.19285	178.60006	119.40247	89.80367	3
20	2054.12952	1027.56840	685.38136	514.28784	H	255.14517	128.07622	85.71991	64.54175	2
21					V	118.08626	59.54677	40.03360	30.27702	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_3.raw #1707 RT: 14.20
FTMS, HCD@40.00, z=+4, Mono m/z=543.55914 Da, MH+=2171.21474 Da, Match Tol.=0.02 Da



Sequence: PAPTHVRSPQPPPP, Charge: +3, Monoisotopic m/z: 493.26672 Da (-1.83 mmu/-3.7 ppm), MH+: 1477.78562 Da, RT: 18.10 min,
Identified with: Sequest HT (v1.3); XCorr:1.47, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

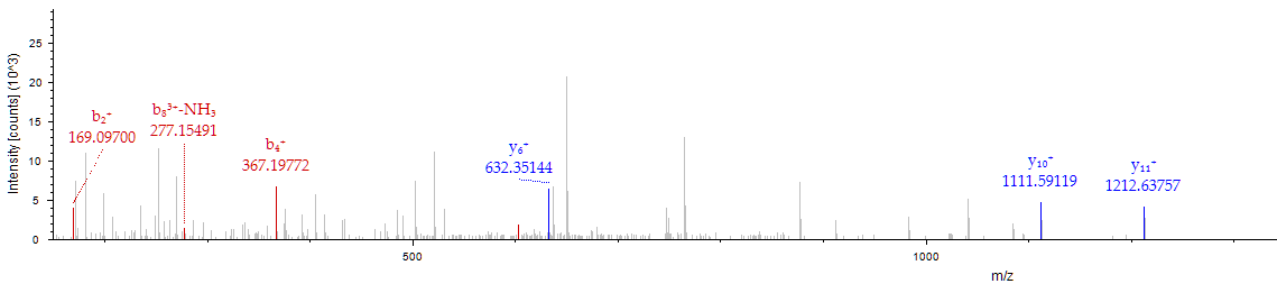
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	b^{3+}	Seq.	y^+	y^{2+}	y^{3+}	#2
1	98.06005	49.53366	33.35820	P				14
2	169.09717	85.05222	57.03724	A	1380.73832	690.87280	460.91762	13
3	266.14994	133.57861	89.38816	P	1309.70120	655.35424	437.23858	12
4	367.19762	184.10245	123.07072	T	1212.64843	606.82785	404.88766	11
5	504.25653	252.63190	168.75703	H	1111.60075	556.30401	371.20510	10
6	603.32495	302.16611	201.77983	V	974.54184	487.77456	325.51880	9
7	759.42607	380.21667	253.81354	R	875.47342	438.24035	292.49599	8
8	846.45810	423.73269	282.82422	S	719.37230	360.18979	240.46228	7
9	943.51087	472.25907	315.17514	P	632.34027	316.67377	211.45161	6
10	1071.56945	536.28836	357.86133	Q	535.28750	268.14739	179.10068	5
11	1168.62222	584.81475	390.21226	P	407.22892	204.11810	136.41449	4
12	1265.67499	633.34113	422.56318	P	310.17615	155.59171	104.06357	3
13	1362.72776	681.86752	454.91410	P	213.12338	107.06533	71.71264	2
14				P	116.07061	58.53894	39.36172	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #2333 RT: 18.10
FTMS, HCD@40.00, z=+3, Mono m/z=493.26672 Da, MH+=1477.78562 Da, Match Tol.=0.02 Da



Sequence: VFPPGPYHPFPNYIR, Charge: +2, Monoisotopic m/z: 900.96155 Da (-3.14 mmu/-3.48 ppm), MH+: 1800.91582 Da, RT: 25.30 min,
Identified with: Sequest HT (v1.3); XCorr:0.58, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-NH_3; y; y-NH_3

Fragment Matches

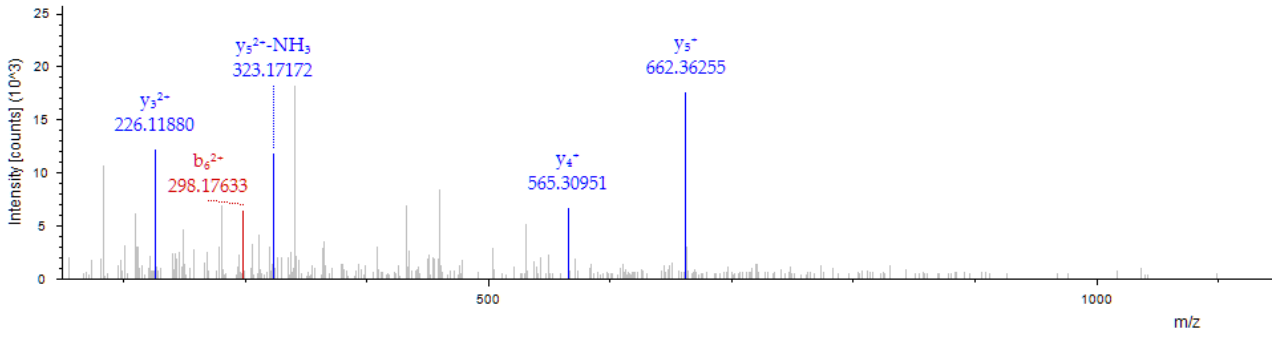
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	100.07570	50.54149	V			15
2	247.14412	124.07570	F	1701.85367	851.43047	14
3	344.19689	172.60208	P	1554.78525	777.89626	13
4	441.24966	221.12847	P	1457.73248	729.36988	12
5	498.27113	249.63920	G	1360.67971	680.84349	11
6	595.32390	298.16559	P	1303.65824	652.33276	10
7	758.38722	379.69725	Y	1206.60547	603.80637	9
8	895.44613	448.22670	H	1043.54215	522.27471	8
9	992.49890	496.75309	P	906.48324	453.74526	7
10	1139.56732	570.28730	F	809.43047	405.21887	6
11	1236.62009	618.81368	P	662.36205	331.68466	5
12	1350.66302	675.83515	N	565.30928	283.15828	4
13	1513.72634	757.36681	Y	451.26635	226.13681	3
14	1626.81041	813.90884	I	288.20303	144.60515	2
15			R	175.11896	88.06312	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #3383 RT: 25.30
FTMS, HCD@40.00, z=+2, Mono m/z=900.96155 Da, MH+=1800.91582 Da, Match Tol.=0.02 Da



Sequence: PRTLQP, P6-Amidated (-0.98402 Da)
Charge: +2, Monoisotopic m/z: 355.71967 Da (+0.62 mmu/+1.74 ppm), MH+: 710.43205 Da, RT: 69.16 min,
Identified with: Sequest HT (v1.3); XCorr:0.64, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da

Fragment Matches

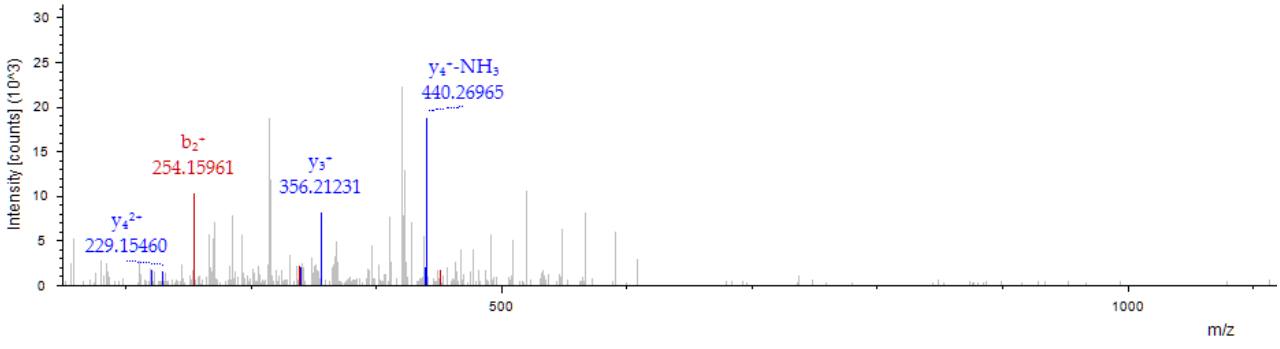
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^{+}	b^{2+}	Seq.	y^{+}	y^{2+}	#2
1	98.06005	49.53366	P			6
2	254.16117	127.58422	R	613.37805	307.19266	5
3	355.20885	178.10806	T	457.27693	229.14210	4
4	468.29292	234.65010	L	356.22925	178.61826	3
5	596.35150	298.67939	Q	243.14518	122.07623	2
6			P-Amidated	115.08660	58.04694	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #9384 RT: 69.16
FTMS, HCD@40.00, z=+2, Mono m/z=355.71967 Da, MH+=710.43205 Da, Match Tol.=0.02 Da



Sequence: LQEQEELENYIEH, Charge: +2, Monoisotopic m/z: 837.38324 Da (-3.09 mmu/-3.69 ppm), MH+: 1673.75920 Da, RT: 17.35 min,
Identified with: Sequest HT (v1.3); XCorr:0.64, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

Fragment Matches

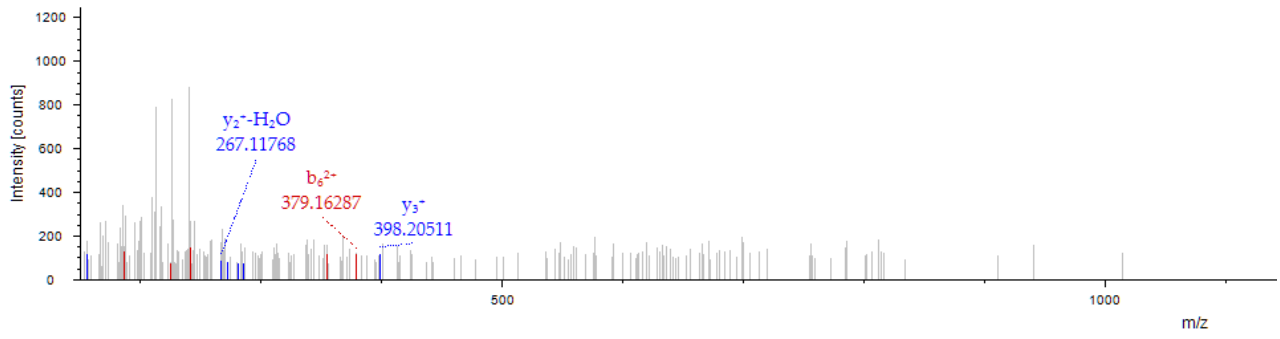
Value Type: Theo. Mass [Da]

Ion Series | Neutral Losses | Precursor Ions

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09135	57.54931	L			13
2	242.14993	121.57860	Q	1560.68130	780.84429	12
3	371.19253	186.09990	E	1432.62272	716.81500	11
4	499.25111	250.12919	Q	1303.58012	652.29370	10
5	628.29371	314.65049	E	1175.52154	588.26441	9
6	757.33631	379.17179	E	1046.47894	523.74311	8
7	870.42038	435.71383	L	917.43634	459.22181	7
8	999.46298	500.23513	E	804.35227	402.67977	6
9	1113.50591	557.25659	N	675.30967	338.15847	5
10	1276.56923	638.78825	Y	561.26674	281.13701	4
11	1389.65330	695.33029	I	398.20342	199.60535	3
12	1518.69590	759.85159	E	285.11935	143.06331	2
13			H	156.07675	78.54201	1

Fragment Spectrum

Extracted from: C:\Users\COMPUTER\Desktop\Analisi PD\Cri_VGF_top down\Rat_Fipronil_nanoESI\250723_Rat_striato_1.raw #2231 RT: 17.35
FTMS, HCD@40.00, z=+2, Mono m/z=837.38324 Da, MH+=1673.75920 Da, Match Tol.=0.02 Da



ethical statement

Rats were handled once daily to avoid stress induced by the experimental procedures during the experimental session and to familiarize them with the experimental operators. All national and institutional guidelines for animal care and use were followed. The care and use of the animals was approved by the American Physiological Society and the EEC Council Directive of 24 November 1986 (86/609) and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari (D.P.R. 116/92).

funding statement

This work was supported by the University of Cagliari, no details are available.

VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS

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Abstract

Background. Dopamine is reduced in the brain of rats treated with fipronil, a broad-spectrum insecticide. VGF (no acronym) is a neurotrophin-inducible protein expressed as the 75kDa form (precursor or pro-VGF) or its truncated peptides. VGF immunostaining has been revealed using an antibody against the C-terminal nonapeptide of the rat pro-VGF in the nerve terminals of the rat substantia nigra, where it was reduced after 6-hydroxydopamine treatment. It is unknown whether pro-VGF and/or its shortened peptides are present in these neurons. Therefore, the aim of this study was first to determine which types of VGF are expressed in the normal substantia nigra (and striatum) and then to determine VGF modulations and whether they occur in parallel with locomotor changes after fipronil injection. Methods. Rats were divided into two groups that received a unilateral intranigral infusion of either vehicle (i.e., dimethyl sulfoxide, DMSO) or fipronil (25 µg), and then were tested for locomotor activity. An untreated group of rats (n=4) was used for identification of the VGF fragments using high performance liquid chromatography-mass spectrometry and western blot, while changes in treated groups (fipronil *vs* DMSO, each n=6) were investigated by immunohistochemistry using an antibody against the rat pro-VGF C-terminal nonapeptide in parallel with the anti-tyrosine hydroxylase antibody. Results. In untreated rats, the VGF C-terminal antibody identified mostly a 75kDa band in the substantia nigra and striatum, supporting the finding of high-resolution mass spectrometry, which revealed fragments covering the majority of the pro-VGF sequence. Furthermore, several shortened VGF C-terminal forms (varying from 10 to 55 kDa) were also found by western blot, while high-resolution mass spectrometry revealed a C-terminal peptide overlapping the immunogen used to create the VGF antibody in both the substantia nigra and the striatum. In the substantia nigra of fipronil-treated rats, immunostaining for tyrosine hydroxylase and VGF was reduced compared to DMSO rat group, and this was related with significant changes in locomotor

activity. Fipronil has the ability to modulate the production of pro-VGF and/or its C-terminal truncated peptides in the nigrostriatal system indicating its intimate interaction with the dopaminergic mechanisms and implying a potential function in modulating locomotor activity.

Key words

Fipronil, Parkinson's disease, VGF, degeneration, nigrostriatal mechanisms, dopamine

1.INTRODUCTION

Parkinson's disease (PD) is a neurological disorder characterized by progressive degeneration of dopaminergic neurons in the substantia nigra (SN), resulting in dopamine deficiency with deregulation of a variety of substances/neurotransmitters (Kalia and Lang, 2015). Environmental factors appear to play an important role in the development of PD. There is an association between PD and pesticides in different exposure settings (industrial, agricultural, residential: Freire and Koifman 2012; Islam et al., 2021; Anirudhan et al., 2023). The staining of tyrosine hydroxylase (TH), the enzyme responsible for dopamine synthesis, is reduced in the SN of rats microinjected with fipronil, a broad-spectrum insecticide also used in veterinary medicine (Park et al., 2016). This reduction paralleled changes in motor activity and nociception due to degeneration of nigrostriatal dopaminergic neurons (Bharatiya et al., 2020a). In addition, chronic oral fipronil treatment for 21 days significantly reduced dopamine and its metabolites in most striatal areas, including the nucleus accumbens and SN (Bharatiya et al., 2020b). The VGF gene (not abbreviated), which is regulated by nerve growth factor (NGF) in PC12 cells and cultured cortical neurons (Salton et al., 1991), encodes a VGF precursor protein (or pro-VGF). Pro-VGF has a molecular weight (MW) of 75kDa and consists of 617/615 aa in rat/mouse and human, respectively, with >85% identity (minor sequence differences between rat/mouse and human) (Ferri et al., 2011). It can give rise to a variety of truncated peptides different in length and biological activity, expressed in rat and human brain (Noli et al, 2017; Cocco et al., 2010), but also in rat/mouse and human blood (Noli et al, 2017; D'Amato et al., 2015). Truncated peptides include the so-called TLQP family, which consists of pleiotropic neuropeptides possibly involved in various physiological processes (Noli et al., 2020; Corda et al, 2021, Lewis et al., 2017, Fairbanks et al., 2014, Skorput et al., 2018) and the NAPP-19, identified in human blood (D'Amato et al., 2015). In the SN, using an antibody against the nonapeptide at the C-terminal (C-t) of the rat pro-VGF, staining was found in a large number of neuron terminals, containing glutamic acid decarboxylase (GAD) and Substance P. Staining of VGF decreased in the SN of 6-

hydroxydopamine-(6-OHDA) treated rats, but it was restored by levodopa (L-dopa) treatment (Cocco et al., 2020). These results are consistent with those obtained in PD patients. Indeed, the levels of the VGF C-t peptides in PD patients were analyzed by a home-made competitive enzyme-linked immunosorbent assay using an antibody directed against the nonapeptide at the C-t of the human pro-VGF. Blood samples were collected from patients at the time of diagnosis (drug-free, n = 23) or after dopamine replacement (n = 40) and compared with age-matched controls (n = 21) (Cocco et al., 2020). A strong decrease (>50%) was observed in drug-free patients, whereas long-term L-dopa treatment caused an increase in the VGF levels. VGF C-t levels were also correlated with disease duration, L-dopa equivalent dose and severity of the olfactory dysfunction. The VGF changes observed in the blood of PD patients have been verified in other body fluids, such as cerebrospinal fluid (CSF) and urine, using liquid chromatography-tandem mass spectrometry in data-independent acquisition mode (Rotunno et al., 2020; Virreira Winter et al., 2021). In CSF, downregulation was observed for peptides containing the C-t portion, whereas in urine downregulated peptides covered most of the VGF sequence (Karayel et al., 2022). Because it is unclear which VGF forms are expressed specifically in the nigrostriatal regions, the goal of this study was to first identify which types of VGF are present in the SN and CPu, and then to determine the possible VGF response to fipronil intranigral microinjection and whether it is associated with locomotor activity.

2. MATERIALS AND METHODS

2.1. Animal care and use

Adult male Sprague-Dawley rats (kindly provided by the Department of Neuroscience, University of Cagliari) weighing 250-300 g (at the beginning of the experiments) were used in this study. The animals were housed in groups of 4 per cage and maintained under standard conditions with a 12-h light/dark cycle at room temperature (22 ± 2°C, 60 ± 5% humidity). Standard pellet chow and tap water were provided ad libitum throughout the study. Rats were handled once daily for at least 10 days before starting the experiments to avoid stress induced by the experimental procedures and to familiarize them with the operators. All national and institutional guidelines for animal care and use were followed. The care and use of the animals was approved by the American Physiological Society and the EEC Council Directive of 24 November 1986 (86/609) and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari (D.P.R. 116/92).

2.2 Experimental timeline of behavioral studies

The assessment of potential effects to locomotor behavior in rats that received an intranigral injection of either vehicle (i.e., DMSO) or fipronil was conducted with minor changes as already described (Angioni et al., 2016; Bharatiya et al., 2020a; Sanna et al., 2021). Briefly, rats were handled daily for 10 days prior to the start of the studies to avoid stress caused by manipulation during the experimental sessions. At the end of this period, each rat underwent one habituation session that lasted for two hours in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Twenty-four hours later, the rats were tested in order to obtain basal values of motor activity and prepare the experimental groups, that was made by assigning each rat to the vehicle or fipronil group in a counterbalanced manner to avoid possible differences between groups in the level of basal activity of the rats. One group (n=12) received a unilateral intranigral infusion of DMSO, while the other group (n=12) was intranigrally injected with fipronil (see below). Finally, 15 days after the intranigral microinjection, each rat was tested for locomotor activity and, thereafter, rats were perfused, and brains collected for the *ex vivo* immunohistochemistry (IHC) assays (see Figure 1).

2.3 Fipronil microinjections into the SN

Rats (n=24) were positioned in a Stoelting stereotaxic apparatus under isoflurane anaesthesia (1.5-2.0%) and a unilateral microinjection of DMSO (1 µL) or fipronil (25 µg/1 µL; purchased from Sigma Aldrich, Düsseldorf, Germany) was performed at the SN coordinates (AP: -5.3 mm; ML: -2.0 mm; DV: -8.0 mm) (Paxinos & Watson, 2004) over a period of 1 minute by using a 10 µL Hamilton microsyringe mounted on the holder of the Stoelting stereotaxic apparatus. The needle of the microsyringe was left in the injection site for a further 3 minutes to allow better diffusion of the injected solution, before being slowly withdrawn (Bharatiya et al., 2020a). To verify the injection site in the SN, selected brain slices at the level of the nigral area were stained with neutral red solution and examined by a contrast-phase microscope. Only rats in which the track of the microinjection needle was correctly positioned in the SN were included in the statistical analysis of the results.

2.4 Locomotor activity

Rats (n=24) were individually tested for motor activity under standardised environmental conditions in a soundproof room with a light level of 30 lux using a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage (42 cm x 42 cm x 63 cm) had two sets of 16 photocells arranged at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor, and another set of 16 horizontal beams whose height was adapted to the size of the animals (20 cm). Horizontal and vertical activities were measured as the total number of consecutive infrared beam breaks (counts) in the

horizontal or vertical sensors, while central time was quantified as the number of seconds the animal spent in the central part of the arena during the experiment. Parameters were recorded every 5 minutes, beginning immediately after the animals were placed in the experimental cage, for a test period of 30 minutes.

2.5 VGF C-t antibody

The sequence His615-Arg616-Pro617 is located at the rat pro-VGF C-t. Therefore, we produced the VGF C-t antibody by immunization against the corresponding nonapeptide as antigen conjugated to bovine thyroglobulin via an additional D-tyrosine. Our VGF C-t antibody, has been shown to recognize the pro-VGF in pancreas (Cocco et al., 2007), stomach (Brancia et al., 2010) and adrenal gland (D'Amato et al., 2008), and was affinity purified by overnight incubation (4°C) in a disposable polypropylene column (Thermo Scientific) containing the VGF C-t cysteine-octapeptide covalently immobilized on a sulfolink coupling resin (Thermo Fisher Scientific). After several rinses with phosphate buffer saline (PBS) 0.5 M, the C-t antiserum was eluted in glycine-HCl 1M, pH 2.5 and validated by (i) absorption experiments performed by overnight incubation on normal SN sections with the rat nonapeptide used for immunization in a range of concentrations (0.01-100 mol/L, at 4°C) (ii) comparison with two purchased C-t antibodies raised against human C-t sequences overlapping our immunogen (# PA5-20523 and # PA5-63081), guaranteed for reactivity in human, rat and mouse, and both validated by western blot (WB) for their reactivity to pro-VGF (the PA5-63081 was validated using a VGF knockout cell line generated by CRISPR-Cas9) (iii) WB using a cell line derived from a rat adrenal medullary pheochromocytoma (PC12) grown with ($PC12^{+}$) or without ($PC12^{-}$) NGF in Roswell Park Memorial Institute (RPMI) medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum, as previously described (Possenti et al. 1989; Greene and Tischler, 1976). If necessary, 100 ng/mL NGF ($PC12^{+}$) was added 24 hours before cell harvest. Cells were harvested in PBS and lysed in sustainable development solution (SDS) sample buffer.

2.6 VGF peptide identification

The VGF forms were identified using a group of untreated rats (n= 4), by high performance liquid chromatography-mass spectrometry and WB.

2.6.1 WB analysis

WB was performed on SN, CPu, accumbens and hypothalamus samples of untreated rats (n=4 each) and either $PC12^{+}$ or $PC12^{-}$. From each fresh brain, coronal slide samples (encompassing the interested areas) were first obtained using a cooled rat brain matrix through razor blades, and then CPu, accumbens and hypothalamus samples were obtained from the slides using

punches of 3, 4 and 5 mm dimensions as appropriate, following, under microscopy, the coordinates of the Paxinos Atlas of the rat brain (Paxinos and Watson, 2007).
Brain samples were placed in a 10 ml/g extraction solution (PBS plus 5 ul/mL protease inhibitor cocktail: Sigma P8340) and immediately homogenized with ultraturrax for one minute. Samples were kept on ice for 10 minutes, then samples were boiled for a further 10 minutes, cooled and centrifuged at 3,000 rpm for 15 minutes at 4°C. The BCA protein assay kit (Thermo Scientific) was used to measure total protein concentration. Proteins were diluted in 2x SDS buffer (Origene) to load 20 micrograms of each sample, heated to 95°C for 5 minutes and subjected to SDS-PAGE using a precast polyacrylamide gradient gel (NuPAGE 4 to 12% Bis-Tris Mini protein Gel, Thermo Fisher Scientific) in a mini gel tank (Thermo Fisher Scientific) for 20 minutes at 200 volts. Internal MW standards (PageRuler Plus prestained protein ladder 10 to 250KDa, Thermo Scientific) were run in parallel. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Hybond-P, GE Healthcare) for 1 hour at 20 volts. Blots were blocked by immersion in 50 mM Tris-base and 150 mM sodium chloride (TBS) containing 0.01% Tween-20 (TBS-T) and 5% bovine serum albumin for 1 hour at room temperature. Incubation with rabbit anti-VGF C-t primary antiserum (1:3,000 dilution in TBS containing 5% bovine serum albumin and 0.02% NaN3) was performed overnight at 4°C. The next day, the blots were rinsed four times with TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted 1:10,000 in TBS-T for 1 hour at room temperature. After several washes with TBS-T, protein bands were developed using Thermo Scientific Pierce Enhanced Chemiluminescence WB Substrate. ImageQuant LAS-4000 was used for chemiluminescence detection.

2.6.2 Nano-reversed phase high-performance liquid-chromatography (nano-RP-HPLC) - high-resolution Electron Spray Ionization-Mass Spectrometry (HR-ESI-MS)

For top-down nano-RP-HPLC-high resolution ESI-MS/MS analysis and VGF peptide identification, all chemicals and reagents used were purchased from Sigma Aldrich (St. Louis, MO, USA). Analysis was performed on protein extracts from SN and CPu from the untreated rat group (2 for SN and 3 for CPu). Protein extraction was performed in PBS with protease inhibitor cocktail as previously described for WB. For each sample, the homogenised tissues were centrifuged and the clear supernatant containing proteins was quantified using the BCA protein assay kit (Thermo Scientific) prior to lyophilisation. The lyophilised samples were then resuspended in 0.1% formic acid (FA) to a final concentration of 0.1 µg/µL and 10 µL were analysed on an Ultimate 3000 Nano System HPLC (Thermo-Fisher Scientific,

Sunnyvale, CA) coupled to an LTQ Orbitrap XL (Thermo-Fisher Scientific, San Jose, CA, USA). The Easy Spray reverse phase nano-column (250 mm x 75 μm inner diameter, Thermo Fisher Scientific) was a C18 with 2 μm beads and elution was achieved with aqueous solvent A (0.1% FA) and aqueous solvent B (0.1% FA, 80% ACN v/v) in 90 min at a flow rate of 0.3 μL/min with the following gradient: 0-3 min at 4%B, 3-10 min 4-20%B, 10-60 min 20-50%B, 60-90 min 50-80%B. The mass spectrometer was operated at 1.5 kV in data dependent acquisition mode with the capillary temperature set at 275°C and the S-Lens RF level set at 68%. Full MS experiments were performed in positive ion mode from 350 to 2000 m/z with a resolution of 120000 (at 400 m/z). The 5 most intense ions were subjected to the high collision dissociation (HCD) fragmentation with settings of 40% of normalised collision energy for 10 ms, isolation width of 2 m/z and activation q of 0.25. Characterisation of naturally occurring intact peptides of VGF was performed using Proteome Discoverer (PD) software (version 1.4, Thermo-Fisher) with the SEQUEST HT cluster search engine (University of Washington, licensed to Thermo Electron Corporation, San Jose, CA) against the target *rattus norvegicus* VGF sequence obtained from the Uniprot KB database. The database search parameters were N-terminal pyroglutamic acid residue and C-terminal amidation as dynamic modifications. The peptide mass tolerance was set to 10 ppm and the fragment ion mass tolerance was set to 0.02 Da. Peptides were filtered for high confidence and a minimum length of 6 amino acids; FDR settings were 0.01 (strict) and 0.05 (relaxed).

2.7 Brain staining

Rats were deeply anaesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused-fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.2–7.4. Brains were rapidly removed, washed overnight in PBS containing 7% sucrose and 0.01% NaN3, oriented in aluminium foil moulds in cryo-embedding medium (Cocco et al., 2003) and frozen in melting freon (cooled with liquid nitrogen). Coronal cryosections (10 μm) encompassing the entire SN and/or CPu obtained from the midbrain (from the section with AP≈-6.5 to the section with AP≈-4.5) were collected on poly-L-lysine-coated slides and stored in the vapour phase of a liquid nitrogen tank until use. IHC investigations were performed on 12 treated rats, with 6 animals from each group (fipronil and DMSO) chosen. The remaining rats (out of a total of 24 treated rats) were not chosen because their brains were clearly not properly perfused (brains still showed red vassels, thus IHC was not conducted) or were only partially perfused, making it difficult to detect positive labelling when performing IHC. IHC was carried out using our rabbit anti-VGF C-t antibody, (1:500), the sheep anti- TH (1:600;) and the two commercial VGF C-t antibodies, (# PA5-20523; against 17 aa at the C-t and PA5-63081

against 100 aa at the C-t); all diluted in PBS containing 30 ml/L normal donkey serum, 30 ml/L normal rat serum and 0.02 g/L NaN3. Sections were incubated overnight in a humidity chamber with the primary antibodies, while the appropriate species-specific donkey secondary antibodies conjugated with either Cy3 or Cy2 were used to detect primary antibody reactivity. Slides were coverslipped with PBS-glycerol (40%), observed and photographed using BX41 and BX51 fluorescence microscopes (Olympus, Milan, Italy) equipped with FujiS3 Pro digital cameras (Fujifilm, Milan, Italy). Routine controls included sequential substitution of each antibody with PBS, use of pre-immune or non-immune sera, and testing of each secondary antibody with its respective non-relevant primary antibody. For semi-quantitative determination of TH and VGF immunofluorescence signal, the FIJI image processing package based on ImageJ (NIH) was used. Briefly, 4 sections/animal/group/areas (SN or striatum) were selected, images were taken under standard exposure conditions with the S3 Pro digital camera (magnification: 4x) to cover the entire SN, including the pars compacta and reticulata. The RGB TIFF images were first converted to 8-bit greyscale, then the boundaries of the SN were manually traced by the user, and finally the background fluorescent signal was removed by a manual thresholding process. The mean SD of the optical density (OD) of binding of each TH and VGF antibody was then calculated and used for statistical analysis for each group of animals.

2.8 Statistical analysis

According to the 3Rs principles, we aim to minimize the number of animals used. To this scope, sample size calculations were performed before starting the experiments to ensure adequate experimental group numbers to be used in the study. Based on these calculations, it was expected that a number of 12 rats would be sufficient to detect significant differences between groups in the locomotor activity tests (t test, effect size d = 1.2, power (1-β) = 0.80, α = 0.05), while a number of 6 rats/group would be sufficient to detect significant differences between groups in the IHC assays (t test, effect size d = 2, power (1-β) = 0.80, α = 0.05). These calculations have been done based on prior studies using similar protocols (see for instance, Angioni et al., 2016; Bharatiya et al., 2020a,b; Sanna et al., 2021) and were carried out by using the software G*Power 3.1 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologieund-arbeitspsychologie/gpower). Behavioral data were expressed as mean ± SEM and analyzed by Student's t-test (in case of the analyses of 30 minutes total counts) or repeated measures two-way ANOVA (in case of the analyses of fractional 5 minutes counts) with the Treatment as a between-subject factor and the 5 minutes Time fractions as a within-subject repeated measure. When ANOVA detected significant effects of main factors or interactions post-hoc comparisons

were performed using Bonferroni's corrected pairwise contrasts. Before running the tests, normal distribution of data was ascertained by the Shapiro-Wilk test and homogeneity of variances was verified by the Bartlett test. The analyses were carried out with PRISM, Graph Pad 8 Software (San Diego, USA) with the significance level set at $p < 0.05$.

The OD collected from IHC experiments was statistically analyzed using Microsoft Excel StatistiXL software. For each experimental set, the normality of the data distribution was preliminarily checked using the goodness-of-fit test. The resulting p-values were > 0.05 in all cases; therefore, the following parametric tests were applied. Sample variances were remeasured using the F-test for equality of variance; therefore, individual or pooled variances were used for the two-tailed Student's t-test. P values < 0.05 were considered significant.

3. RESULTS

3.1 VGF fragments in the SN and striatum of untreated rats

Our VGF antibody detected a 75kDa band compatible with the pro-VGF using WB performed with brain samples (SN, CPu, accumbens and hypothalamus) each from 4 untreated rats (Fig. 2). The same band was also labelled using $PC12^{+}$, but not with $PC12^{-}$. These results are consistent with the top-down nano-RP-HPLC-high resolution ESI-MS/MS analysis done using untreated rats (n=2 of SN and 3 of CPu), which identified intact peptides (numbering seven in the SN and seventeen in the striatum), largely covering the VGF sequence. Importantly, among the fragments containing the C-t portion, one of 13 aa was characterized in the CPu (LQEQEELENYIEH peptide: $VGF_{599-611}$), whereas a longer peptide of 17 aa with the highest overlap with the immunogen used to generate our anti-VGF antibody (EQEELENYIEHVLLHRP: $VGF_{601-617}$) was identified in the SN. Although other bands smaller than pro-VGF (55, 35, 15 and 10 kDa) were also detected by WB (probably because they contain the C-t portion), these forms were not the same as those identified by HPLC analysis because of the different detection limits of the techniques. The complete list of peptides identified in both tissues is shown in Fig. 3 and reported in detail in the supplemental material (Tables S1 and S2). Comparing the two brain areas, the majority of peptides expressed in the SN are also found in the CPu, including the NAPP, whereas the CPu contains peptides that are exclusively expressed in this area, including the TLQP.

3.2 Immunostaining in treated rats

Immnuofluorescence was performed in DMS- and fipronil-treated rats and semi-quantitative statistical changes were investigated (n=6 each; Fig.4 a–l). Using SN sections of DMSO treated rats, in both injected and uninjected sides, TH staining was observed as expected, and our home-made VGF antibody labeled axon terminals possibly containing GAD and

Substance P, as previously reported (Cocco et al., 2020), but did not label dopaminergic perikarya. Using the SN sections for absorption experiments, almost complete prevention of labelling of our home-made VGF antibody was found at the high peptide concentration, while some brightness intensity was shown at the other peptide concentrations. The same labelling was also observed using the two commercial anti-VGF C-t antibodies raised against two C-t peptides partially overlapping our immunogen (Fig. 5). Using SN from unilaterally fipronil treated rats, the uninjected sides were comparable to both the DMSO -injected (Fig. 4 a, d) and -uninjected sides, with the TH antibody labeling cell bodies and dendrites as expected (Fig. 4, a), and our VGF C-t antibody staining neuronal terminals (but not cell bodies) within both the pars compacta and reticulata (Fig. 4, d). Instead, in the SN of the fipronil treated rats the injected sides (Fig. 4 b, e) revealed a decrease in TH immunostaining with fewer positive cell bodies and dendrites compared to the DMSO-injected (Fig. 4, b vs a), DMSO-uninjected and fipronil-uninjected sides. The decrease in TH immunostaining was more pronounced around the injection side, but remained reduced throughout the different sections examined. Correspondingly, both the intensity and brightness of the VGF C-t staining was reduced on the fipronil injected sides compared to DMSO -injected (Fig. 4 e *vs.* d, respectively) DMSO-uninjected and fipronil-uninjected sides and the reduction was more pronounced around the injection, but, as observed for TH, it remained reduced throughout the different sections examined. In the striatum of DMSO treated rats, in both sides VGF staining was observed in many areas of the brain, including the CPu, accumbens, and the cortex where cell bodies were labelled within the different layers, as well as the hippocampus and hypothalamus. Because neurons in the SN containing both GAD and substance P are known to be CPu projections, we focused on VGF labeling inside CPu. However, in DMSO treated rats (both sides), fipronil-injected and fipronil-uninjected sides, both VGF (Fig. 4 g and h, DMSO and fipronil injected sides, respectively) and TH (Fig. 4 j and k, DMSO and fipronil injected sides, respectively) staining were almost identical. VGF staining was distributed throughout the entire CPu and identified primarily in neuronal terminals and rarely in cell bodies, making it difficult to characterize a single location as the side of SN projections (other techniques should be used to properly describe it). To determine semi-quantitative alterations in immunofluorescence signals, we examined the OD for TH and VGF in both SN and CPu, comparing the fipronil to DMSO -injected sides. In the SN, OD for TH- (Fig. 4, c) and for VGF C-t- labelling (Fig. 4, f) showed a significant reduction after fipronil treatment ($p<0.05$). Instead, in the CPu, the TH (Fig. 4, i) and VGF (Fig. 4, l) staining did not differ when the entire CPu was selected in ImageJ to see if the overall OD changed in fipronil compared to DMSO -injected sides.

3.3 Locomotor activity after FPN injection into the SN

As reported in Fig. 6, rats unilaterally injected into the SN with a dose of 25 μg of fipronil displayed significant differences in locomotor activity 15 days after the injection compared to vehicle (i.e., DMSO) treated rats. In particular, fipronil-treated rats displayed a significant increase in horizontal activity; accordingly, two-way ANOVA of the counts referred to the 5 min fractions revealed a significant effect of treatment [$F(1, 22) = 9.48$, $p = 0.005$] (Fig. 6, a) and this difference was also confirmed by the t test performed on the 30 minutes total counts ($p = 0.005$) (Fig. 6, b). At variance, in the case of vertical activity, two-way ANOVA detected a significant treatment x time interaction [$F(5, 110) = 4.42$, $p = 0.001$] (Fig. 6, c) indicating slightly lower values in FPN-treated rats when compared to vehicle-treated ones. However, this difference was not more detectable when analyzing the 30 minutes total counts by the t test (Fig. 6, d). Finally, no significant differences between groups were observed in the center time, nor by the ANOVA on 5 minutes fractions neither by the t test on total counts (Fig.6, e, f).

4. DISCUSSION

The results of our studies showed that fipronil injection induced not only a reduction in TH, as previously reported (Park et al., 2016; Bharatiya et al., 2020a), but also in VGF staining, in parallel with changes in locomotor activity, that were detectable 15 days after the intranigral injection of the pesticide. As for our anti-VGF antibody, it has previously been used in SN (Cocco et al., 2020), but here we made a further effort to better characterise it using affinity purification, WB analysis (also using PC12 cells), and commercial antibodies against C-t sequences partially overlapping our immunogen and validated for their reactivity against pro-VGF. WB analysis confirmed that our VGF antibody recognised pro-VGF in the SN and striatum. These data are consistent with HPLC-MS which, although unable to recognise the 75kDa pro-VGF form, detected several fragments covering a large part of the VGF sequence. Among the VGF peptides, those containing C-t sequences partially overlapping with the immunogen used to generate our VGF antibody were identified in the striatum and SN. To the best of our knowledge, this is the first report of the identification of multiple naturally occurring VGF fragments in the SN using HPLC-MS, while some VGF fragments were previous found in the rat striatum (Karlsson et al., 2013) and striatum secretome (Bernay et al., 2009). Thanks to this finding, specific VGF peptides could be used to explore experimental approaches with specific therapeutic purposes using animal models. Furthermore, HPLC-MS, which is independent of antibody reactivity, confirmed the results

obtained by IHC and led to the hypothesis that the VGF changes in the SN under fipronil treatment are related to pro-VGF and/or its C-t truncated peptides.

In our previous investigation (Bharatiya et al., 2020a) we observed that the microinjection of 25 μg FPN into the SN induced 15 days later a decrease of about 40-50% in dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) tissue levels in the striatum, that was paralleled by a decrease to a similar extent of TH immunoreactivity in the SN. Here, although we confirm the changes observed in TH immunoreactivity in the SN, we were unable to detect significant TH immunoreactivity changes in the striatum. These contrasting results may be due to differences in the methods used; in particular, in our previous study we used high pressure liquid chromatography coupled to electrochemical detection (HPLC-ECD) that is more sensitive than the IHC used in the present investigation (on this regard, see also Yuan et al., 2005).

More surprising is the fact that VGF decreased in the SN but not in the striatum. In fact, VGF nerve terminals in the SN are probably projections from the striatum (Bolam and Smith, 1990; Cocco et al., 2020). However, striatal VGF is not only released in the SN but can also be transported into several afferent and efferent fibres, making it difficult to see differences if the change is confined to a small area. Furthermore, the mechanisms by which VGF is involved are not known, so a decrease in VGF within the nigrostriatal pathways could be associated with an increase in other(s) striatal pathways. Importantly, the present findings not only confirm, as in our previous study (Bharatiya et al., 2020a), that direct injection of FPN into the SN induces changes in locomotor activity (i.e., an increase in horizontal activity paralleled by a decrease in vertical activity) associated with dopaminergic degeneration, but also show that these changes can be associated with a decrease in VGF immunoreactivity within the SN. Although further studies are needed to elucidate the putative involvement and role of VGF in regulating pathways with a crucial role in locomotor activity and movement, our results are consistent with previous investigations linking VGF, dopamine and neurodegeneration. Indeed, a similar decrease in VGF was observed in rats treated with 6-hydroxydopamine, which was reversed by L-dopa administration (Cocco et al., 2010). Since the present work shows that environmental factors such as pesticides like fipronil can induce degeneration of dopaminergic neurons but also reduced release of VGF protein, it appears that VGF signalling can be directly regulated by dopamine function and is affected by low dopamine levels independently of the specific pathological processes involved in neurodegeneration, confirming its close relationship with dopaminergic neurotransmission, at least at the level of the nigrostriatal pathways.

However, given to the scarcity of studies on the subject, the involvement of VGF in nigrostriatal circuits is not yet obvious, and more focalized studies are required.
The behavioral alterations in locomotor activity induced by the fipronil microinjection in the SN deserve some additional comment. As recalled above, an increase in horizontal activity together with a slight decrease in the vertical one has been observed after fipronil treatment. There could be several reasons for this discrepancy in the direction of effects between horizontal and vertical activity. One of them relates to the fact that the two parameters quantify different aspects of motor behavior that involve partially different neural pathways. In fact, while horizontal activity (i.e., distance travelled in the horizontal plane) is usually considered an index of general motor activation and arousal and involves not only the activity of the dopaminergic nigrostriatal pathway but also that of the mesolimbic one (see for instance Sharp et al., 1987), vertical activity (expressed by the rearing behavior) is considered a more specific index of active exploration and has been specifically related to the nigrostriatal pathway and striatal dopamine function (see, for instance, Jicha and Salamone, 1991; Cousins et al., 1993). Thus, it is possible that the lesion induced in our rats, inducing a degeneration of the dopaminergic nigral neurons of approximately 60%, with a consequently relatively partial decrease of dopamine in the striatum can lead to a decrease in vertical activity (more sensitive to partial nigrostriatal damage) and, at the same time, to an imbalance in the activity of the pathways involved in the horizontal component of locomotor activity (nigrostriatal and mesolimbic pathways) that lead to an increase other than a decrease in horizontal activity. Support for this notion comes also from the observation that an increase in locomotor activity has been observed 6 days after treatment in rats with lesions of the nigrostriatal dopaminergic neurons induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine given at doses that produce a 50–60% decrease in striatal dopamine content (Ferro et al., 2005), although this difference was no more evident in the same rats after 18 days, pointing out the possibility that time-dependent alterations due to the time-course of the dopamine degeneration could be responsible of the different behavioral effects observed at different time-points in locomotor activity. To note, in the same study 6-OHDA did not induce any significant decreases in horizontal activity at both time-points, though a tendency to decrease rearing behavior was reported after 6 days. Noteworthy, the most part of the studies that observed significant reductions in locomotor activity after the lesion of the nigrostriatal pathway, were conducted inducing bilateral lesions able to provoke up to 80% (or more) dopamine decrease (Deumens et al., 2002) and often injecting the lesioning agent at the level of the medial forebrain bundle (MFB), thus also inducing the lesion of the dopaminergic

mesolimbic pathway. For this reason, this kind of lesions is usually considered as a model of end-stage parkinsonism (see Yuan et al., 2005) and can differ in several aspects from other models where the lesion is of lower entity, as in our case, and that could represent a model of early-stage parkinsonism. Further studies, in which the neurochemical and behavioral monitoring of rats injected with fipronil into the SN will last longer are needed to deeply investigate this possibility. Moreover, it should be also taken into account that at variance from other lesioning agents that are able to induce highly selective lesions of the dopamine system when injected into the SN or in the MFB (such as 6-OHDA), fipronil could also act on other neurotransmitter systems, inducing less selective lesions able to imbalance the activity of the basal ganglia at a more generalized level (see, for instance, Angioni et al., 2016). Finally, both the increase in the horizontal locomotor activity observed in the present study and the entity of the lesion induced in the TH immunoreactivity in the SN (by about 50%) closely resemble the findings reported and extensively discussed in our previous study (Bharatiya et al., 2020a), confirming the reproducibility of the model used. However, at variance from our previous study (Bharatiya et al., 2020a), we detected here a significant time x treatment interaction in the vertical activity data, indicating a slight, though significant, decrease in the vertical component of motor behavior, that did not reach significance (although present) in our previous study. However, this difference was not more significant when analysing the total scores of the 30 minutes test that leads to hypothesize that under the conditions used in this and our previous study this behavioral effect can represent a borderline finding that deserves further attention in next investigations.

5. CONCLUSION

Overall, further studies are needed to better understand the role of the pesticide fipronil as a predisposing agent for the development of PD or other neurodegenerative disorders. The fact that significant impairments in brain monoamine neurochemistry can be observed not only after direct injection of the pesticide into the SN (Bharatiya et al., 2020a), but also after chronic systemic exposure (Bharatiya et al., 2020b), deserves particular attention for the regulation and prevention of those environmental factors that in exposed humans may act as predisposing agents for the development of various neurodegenerative disorders, including PD.

Notably, fipronil can affect not only dopamine secretion but also the generation of pro-VGF and/or specifically VGF C-t truncated peptides in the nigrostriatal system, revealing a potential role in influencing locomotor activity. This could be valuable not only for

diagnostics, as previously proposed (Cocco et al., 2020), but also for experimental treatment methods based on the detected biological peptides.

FIGURE LEGENDS

Fig. 1 Timeline of the experimental procedures

Timeline of the experimental procedures was performed with the cohort of rats dedicated to the behavioral testing of locomotor activity. After a period of ten days of acclimation and daily handling, each rat underwent one habituation session in the motility apparatus that lasted for two hours in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Twenty-four hours later, the rats were tested in order to obtain basal values of locomotor activity and build the experimental groups. Then, the day after, DMSO or fipronil microinjection into the SN was performed. Finally, after a recovery period of 15 days, each rat was individually tested for locomotor activity in order to assess potential effects of the fipronil intranigral microinjection and, thereafter, rats were perfused, and brains collected for the *ex vivo* IHC assays.

Fig. 2 The molecular weight forms recognised by the VGF antibody

A band of approximately 70 kDa, compatible with pro-VGF, was recognised by our anti-VGF C-t antibody in samples of hypothalamus, substantia nigra, caudate-putamen (CPu), nucleus accumbens (n=4 each) and $PC12^{+}$, but not in $PC12^{-}$ (used as negative control); our antibody also recognised other smaller bands compatible with high cleavage of the pro-VGF at its C-terminus. PC12: pheochromocytoma cells, grown up with or without the nerve growth factor ($PC12^{+}$ or $PC12^{-}$, respectively).

Fig. 3 High-resolution MS/MS analysis

Naturally occurring peptides identified by high-resolution MS/MS analysis within the rat pro-VGF sequence in substantia nigra (from 2 untreated rats) and caudate-putamen (from 3 untreated rats). The blue and green arrows indicate peptides from the substantia nigra and caudate-putamen, respectively.

Fig. 4 Changes in treated rats

The TH and VGF antibodies were studied in DMSO-treated (unlesioned) rats *versus* the fipronil-(lesioned) rats (n=6 each group). In the subtantia nigra (a–f), the anti-TH antibody (a) labelled, as expected, several cell bodies and dendrites in the DMSO-treated (unlesioned) side, whereas in the fipronil-(lesioned) side (b) there was a significant reduction in the number of labelled neurons confirmed by a statistically significant decrease in optical density (OD) values (c, OD: 13.5 ± 3.3 *vs.* 47.4 ± 7.1; mean ± SEM; fipronil vs. DMSO sides). Our

anti-VGF antibody labelled axon terminals in the DMSO- treated (unlesioned) side (d), while in the fipronil-(lesioned) side (e) there was a significant decrease in brightness, confirmed by a statistically significant difference in OD values between the two sides (f, OD: 23.7 ± 7.7 *vs* 60.1 ± 7.6 mean ± SD, fipronil vs. DMSO sides). In the caudate-putamen (g–l), no differences were found for TH- or VGF- staining between the DMSO-treated (unlesioned) and fipronil-(lesioned) side (g and j *vs.* h and k, respectively), confirmed by the OD analysis (i, OD for TH:1331±249 and 1192±194 in the DMSO and fipronil lesioned sides, respectively; mean ± SD and l, OD for VGF: 2211±585 and 2005±283 in the DMSO and fipronil lesioned sides, respectively; mean ± SD). TH: tyrosine hydroxylase; scale bars: 50 um (a,b,d,e); 500 um (g,h,j,k).

Fig. 5 VGF Sequences

Sequences of the VGF immunogens used to generate the two commercial antibodies, composed of 100 and 17 aa (Ab_1 and Ab_2: # PA5-63081and PA5-20523, respectively), and our antibody (Ab_3); Ab=antibody; aa=ammino acids.

Fig. 6 Locomotor activity

Horizontal (a and b), vertical (c and d) and centre (e and f) spontaneous locomotor activity of the fipronil- (FPN, 25μg) and dimethyl sulfoxide- (DMSO,1 μL) treated rats. Rats were individually placed inside the apparatus and locomotor activity was recorded for 30 min (6 consecutive fractions of 5 min). On the left (a, c, e) are reported the values related to the individual 5 min recording fractions while on the right (b, d, f) are reported those related to the whole 30 min test. Values are mean ± SEM (n = 12 rats/group; t-test or two-way ANOVA followed by Bonferroni's corrected pairwise comparisons).

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

CCocco, FS contributions to concept/design, EM, GC, BN, MH, AM, BM, CContini, FS, AA, MRM, acquisition of data and data analysis/interpretation, CCocco and FS drafting and critical revision of the manuscript. All authors contributed to the revision of the manuscript and article approval.

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VGF MODICATIONS RELATED TO DOPAMINERGIC NEURODEGENERATION INDUCED BY THE PESTICIDE FIPRONIL IN ADULT MALE RATS

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Abstract

Background. Dopamine is reduced in the brain of rats treated with fipronil, a broad-spectrum insecticide. VGF (no acronym) is a neurotrophin-inducible protein expressed as the 75kDa form (precursor or pro-VGF) or its truncated peptides. VGF immunostaining has been revealed using an antibody against the C-terminal nonapeptide of the rat pro-VGF in the nerve terminals of the rat substantia nigra, where it was reduced after 6-hydroxydopamine treatment. It is unknown whether pro-VGF and/or its shortened peptides are present in these neurons. Therefore, the aim of this study was first to determine which types of VGF are expressed in the normal substantia nigra (and striatum) and then to determine VGF modulations and whether they occur in parallel with locomotor changes after fipronil injection. Methods. Rats were divided into two groups that received a unilateral intranigral infusion of either vehicle (i.e., dimethyl sulfoxide, DMSO) or fipronil (25 µg), and then were tested for locomotor activity. An untreated group of rats (n=4) was used for identification of the VGF fragments using high performance liquid chromatography-mass spectrometry and western blot, while changes in treated groups (fipronil *vs* DMSO, each n=6) were investigated by immunohistochemistry using an antibody against the rat pro-VGF C-terminal nonapeptide in parallel with the anti-tyrosine hydroxylase antibody. Results. In untreated rats, the VGF C-terminal antibody identified mostly a 75kDa band in the substantia nigra and striatum, supporting the finding of high-resolution mass spectrometry, which revealed fragments covering the majority of the pro-VGF sequence. Furthermore, several shortened VGF C-terminal forms (varying from 10 to 55 kDa) were also found by western blot, while high-resolution mass spectrometry revealed a C-terminal peptide overlapping the immunogen used to create the VGF antibody in both the substantia nigra and the striatum. In the substantia nigra of fipronil-treated rats, immunostaining for tyrosine hydroxylase and VGF was reduced compared to DMSO rat group, and this was related with significant changes in locomotor

activity. Fipronil has the ability to modulate the production of pro-VGF and/or its C-terminal truncated peptides in the nigrostriatal system indicating its intimate interaction with the dopaminergic mechanisms and implying a potential function in modulating locomotor activity.

Key words

Fipronil, Parkinson's disease, VGF, degeneration, nigrostriatal mechanisms, dopamine

1.INTRODUCTION

Parkinson's disease (PD) is a neurological disorder characterized by progressive degeneration of dopaminergic neurons in the substantia nigra (SN), resulting in dopamine deficiency with deregulation of a variety of substances/neurotransmitters (Kalia and Lang, 2015). Environmental factors appear to play an important role in the development of PD. There is an association between PD and pesticides in different exposure settings (industrial, agricultural, residential: Freire and Koifman 2012; Islam et al., 2021; Anirudhan et al., 2023). The staining of tyrosine hydroxylase (TH), the enzyme responsible for dopamine synthesis, is reduced in the SN of rats microinjected with fipronil, a broad-spectrum insecticide also used in veterinary medicine (Park et al., 2016). This reduction paralleled changes in motor activity and nociception due to degeneration of nigrostriatal dopaminergic neurons (Bharatiya et al., 2020a). In addition, chronic oral fipronil treatment for 21 days significantly reduced dopamine and its metabolites in most striatal areas, including the nucleus accumbens and SN (Bharatiya et al., 2020b). The VGF gene (not abbreviated), which is regulated by nerve growth factor (NGF) in PC12 cells and cultured cortical neurons (Salton et al., 1991), encodes a VGF precursor protein (or pro-VGF). Pro-VGF has a molecular weight (MW) of 75kDa and consists of 617/615 aa in rat/mouse and human, respectively, with >85% identity (minor sequence differences between rat/mouse and human) (Ferri et al., 2011). It can give rise to a variety of truncated peptides different in length and biological activity, expressed in rat and human brain (Noli et al, 2017; Cocco et al., 2010), but also in rat/mouse and human blood (Noli et al, 2017; D'Amato et al., 2015). Truncated peptides include the so-called TLQP family, which consists of pleiotropic neuropeptides possibly involved in various physiological processes (Noli et al., 2020; Corda et al, 2021, Lewis et al., 2017, Fairbanks et al., 2014, Skorput et al., 2018) and the NAPP-19, identified in human blood (D'Amato et al., 2015). In the SN, using an antibody against the nonapeptide at the C-terminal (C-t) of the rat pro-VGF, staining was found in a large number of neuron terminals, containing glutamic acid decarboxylase (GAD) and Substance P. Staining of VGF decreased in the SN of 6-

hydroxydopamine-(6-OHDA) treated rats, but it was restored by levodopa (L-dopa) treatment (Cocco et al., 2020). These results are consistent with those obtained in PD patients. Indeed, the levels of the VGF C-t peptides in PD patients were analyzed by a home-made competitive enzyme-linked immunosorbent assay using an antibody directed against the nonapeptide at the C-t of the human pro-VGF. Blood samples were collected from patients at the time of diagnosis (drug-free, n = 23) or after dopamine replacement (n = 40) and compared with age-matched controls (n = 21) (Cocco et al., 2020). A strong decrease (>50%) was observed in drug-free patients, whereas long-term L-dopa treatment caused an increase in the VGF levels. VGF C-t levels were also correlated with disease duration, L-dopa equivalent dose and severity of the olfactory dysfunction. The VGF changes observed in the blood of PD patients have been verified in other body fluids, such as cerebrospinal fluid (CSF) and urine, using liquid chromatography-tandem mass spectrometry in data-independent acquisition mode (Rotunno et al., 2020; Virreira Winter et al., 2021). In CSF, downregulation was observed for peptides containing the C-t portion, whereas in urine downregulated peptides covered most of the VGF sequence (Karayel et al., 2022). Because it is unclear which VGF forms are expressed specifically in the nigrostriatal regions, the goal of this study was to first identify which types of VGF are present in the SN and CPu, and then to determine the possible VGF response to fipronil intranigral microinjection and whether it is associated with locomotor activity.

2. MATERIALS AND METHODS

2.1. Animal care and use

Adult male Sprague-Dawley rats (kindly provided by the Department of Neuroscience, University of Cagliari) weighing 250-300 g (at the beginning of the experiments) were used in this study. The animals were housed in groups of 4 per cage and maintained under standard conditions with a 12-h light/dark cycle at room temperature (22 ± 2°C, 60 ± 5% humidity). Standard pellet chow and tap water were provided ad libitum throughout the study. Rats were handled once daily for at least 10 days before starting the experiments to avoid stress induced by the experimental procedures and to familiarize them with the operators. All national and institutional guidelines for animal care and use were followed. The care and use of the animals was approved by the American Physiological Society and the EEC Council Directive of 24 November 1986 (86/609) and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari (D.P.R. 116/92).

2.2 Experimental timeline of behavioral studies

The assessment of potential effects to locomotor behavior in rats that received an intranigral injection of either vehicle (i.e., DMSO) or fipronil was conducted with minor changes as already described (Angioni et al., 2016; Bharatiya et al., 2020a; Sanna et al., 2021). Briefly, rats were handled daily for 10 days prior to the start of the studies to avoid stress caused by manipulation during the experimental sessions. At the end of this period, each rat underwent one habituation session that lasted for two hours in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Twenty-four hours later, the rats were tested in order to obtain basal values of motor activity and prepare the experimental groups, that was made by assigning each rat to the vehicle or fipronil group in a counterbalanced manner to avoid possible differences between groups in the level of basal activity of the rats. One group (n=12) received a unilateral intranigral infusion of DMSO, while the other group (n=12) was intranigrally injected with fipronil (see below). Finally, 15 days after the intranigral microinjection, each rat was tested for locomotor activity and, thereafter, rats were perfused, and brains collected for the *ex vivo* immunohistochemistry (IHC) assays (see Figure 1).

2.3 Fipronil microinjections into the SN

Rats (n=24) were positioned in a Stoelting stereotaxic apparatus under isoflurane anaesthesia (1.5-2.0%) and a unilateral microinjection of DMSO (1 µL) or fipronil (25 µg/1 µL; purchased from Sigma Aldrich, Düsseldorf, Germany) was performed at the SN coordinates (AP: -5.3 mm; ML: -2.0 mm; DV: -8.0 mm) (Paxinos & Watson, 2004) over a period of 1 minute by using a 10 µL Hamilton microsyringe mounted on the holder of the Stoelting stereotaxic apparatus. The needle of the microsyringe was left in the injection site for a further 3 minutes to allow better diffusion of the injected solution, before being slowly withdrawn (Bharatiya et al., 2020a). To verify the injection site in the SN, selected brain slices at the level of the nigral area were stained with neutral red solution and examined by a contrast-phase microscope. Only rats in which the track of the microinjection needle was correctly positioned in the SN were included in the statistical analysis of the results.

2.4 Locomotor activity

Rats (n=24) were individually tested for motor activity under standardised environmental conditions in a soundproof room with a light level of 30 lux using a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage (42 cm x 42 cm x 63 cm) had two sets of 16 photocells arranged at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor, and another set of 16 horizontal beams whose height was adapted to the size of the animals (20 cm). Horizontal and vertical activities were measured as the total number of consecutive infrared beam breaks (counts) in the

horizontal or vertical sensors, while central time was quantified as the number of seconds the animal spent in the central part of the arena during the experiment. Parameters were recorded every 5 minutes, beginning immediately after the animals were placed in the experimental cage, for a test period of 30 minutes.

2.5 VGF C-t antibody

The sequence His615-Arg616-Pro617 is located at the rat pro-VGF C-t. Therefore, we produced the VGF C-t antibody by immunization against the corresponding nonapeptide as antigen conjugated to bovine thyroglobulin via an additional D-tyrosine. Our VGF C-t antibody, has been shown to recognize the pro-VGF in pancreas (Cocco et al., 2007), stomach (Brancia et al., 2010) and adrenal gland (D'Amato et al., 2008), and was affinity purified by overnight incubation (4°C) in a disposable polypropylene column (Thermo Scientific) containing the VGF C-t cysteine-octapeptide covalently immobilized on a sulfolink coupling resin (Thermo Fisher Scientific). After several rinses with phosphate buffer saline (PBS) 0.5 M, the C-t antiserum was eluted in glycine-HCl 1M, pH 2.5 and validated by (i) absorption experiments performed by overnight incubation on normal SN sections with the rat nonapeptide used for immunization in a range of concentrations (0.01-100 mol/L, at 4°C) (ii) comparison with two purchased C-t antibodies raised against human C-t sequences overlapping our immunogen (# PA5-20523 and # PA5-63081), guaranteed for reactivity in human, rat and mouse, and both validated by western blot (WB) for their reactivity to pro-VGF (the PA5-63081 was validated using a VGF knockout cell line generated by CRISPR-Cas9) (iii) WB using a cell line derived from a rat adrenal medullary pheochromocytoma (PC12) grown with ($PC12^{+}$) or without ($PC12^{-}$) NGF in Roswell Park Memorial Institute (RPMI) medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum, as previously described (Possenti et al. 1989; Greene and Tischler, 1976). If necessary, 100 ng/mL NGF ($PC12^{+}$) was added 24 hours before cell harvest. Cells were harvested in PBS and lysed in sustainable development solution (SDS) sample buffer.

2.6 VGF peptide identification

The VGF forms were identified using a group of untreated rats (n= 4), by high performance liquid chromatography-mass spectrometry and WB.

2.6.1 WB analysis

WB was performed on SN, CPu, accumbens and hypothalamus samples of untreated rats (n=4 each) and either $PC12^{+}$ or $PC12^{-}$. From each fresh brain, coronal slide samples (encompassing the interested areas) were first obtained using a cooled rat brain matrix through razor blades, and then CPu, accumbens and hypothalamus samples were obtained from the slides using

punches of 3, 4 and 5 mm dimensions as appropriate, following, under microscopy, the coordinates of the Paxinos Atlas of the rat brain (Paxinos and Watson, 2007).

Brain samples were placed in a 10 ml/g extraction solution (PBS plus 5 ul/mL protease inhibitor cocktail: Sigma P8340) and immediately homogenized with ultraturrax for one minute. Samples were kept on ice for 10 minutes, then samples were boiled for a further 10 minutes, cooled and centrifuged at 3,000 rpm for 15 minutes at 4°C. The BCA protein assay kit (Thermo Scientific) was used to measure total protein concentration. Proteins were diluted in 2x SDS buffer (Origene) to load 20 micrograms of each sample, heated to 95°C for 5 minutes and subjected to SDS-PAGE using a precast polyacrylamide gradient gel (NuPAGE 4 to 12% Bis-Tris Mini protein Gel, Thermo Fisher Scientific) in a mini gel tank (Thermo Fisher Scientific) for 20 minutes at 200 volts. Internal MW standards (PageRuler Plus prestained protein ladder 10 to 250KDa, Thermo Scientific) were run in parallel. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Hybond-P, GE Healthcare) for 1 hour at 20 volts. Blots were blocked by immersion in 50 mM Tris-base and 150 mM sodium chloride (TBS) containing 0.01% Tween-20 (TBS-T) and 5% bovine serum albumin for 1 hour at room temperature. Incubation with rabbit anti-VGF C-t primary antiserum (1:3,000 dilution in TBS containing 5% bovine serum albumin and 0.02% NaN3) was performed overnight at 4°C. The next day, the blots were rinsed four times with TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted 1:10,000 in TBS-T for 1 hour at room temperature. After several washes with TBS-T, protein bands were developed using Thermo Scientific Pierce Enhanced Chemiluminescence WB Substrate. ImageQuant LAS-4000 was used for chemiluminescence detection.

2.6.2 Nano-reversed phase high-performance liquid-chromatography (nano-RP-HPLC) - high-resolution Electron Spray Ionization-Mass Spectrometry (HR-ESI-MS)

For top-down nano-RP-HPLC-high resolution ESI-MS/MS analysis and VGF peptide identification, all chemicals and reagents used were purchased from Sigma Aldrich (St. Louis, MO, USA). Analysis was performed on protein extracts from SN and CPu from the untreated rat group (2 for SN and 3 for CPu). Protein extraction was performed in PBS with protease inhibitor cocktail as previously described for WB. For each sample, the homogenised tissues were centrifuged and the clear supernatant containing proteins was quantified using the BCA protein assay kit (Thermo Scientific) prior to lyophilisation. The lyophilised samples were then resuspended in 0.1% formic acid (FA) to a final concentration of 0.1 µg/µL and 10 µL were analysed on an Ultimate 3000 Nano System HPLC (Thermo-Fisher Scientific,

Sunnyvale, CA) coupled to an LTQ Orbitrap XL (Thermo-Fisher Scientific, San Jose, CA, USA). The Easy Spray reverse phase nano-column (250 mm x 75 µm inner diameter, Thermo Fisher Scientific) was a C18 with 2 µm beads and elution was achieved with aqueous solvent A (0.1% FA) and aqueous solvent B (0.1% FA, 80% ACN v/v) in 90 min at a flow rate of 0.3 µL/min with the following gradient: 0-3 min at 4%B, 3-10 min 4-20%B, 10-60 min 20-50%B, 60-90 min 50-80%B. The mass spectrometer was operated at 1.5 kV in data dependent acquisition mode with the capillary temperature set at 275°C and the S-Lens RF level set at 68%. Full MS experiments were performed in positive ion mode from 350 to 2000 m/z with a resolution of 120000 (at 400 m/z). The 5 most intense ions were subjected to the high collision dissociation (HCD) fragmentation with settings of 40% of normalised collision energy for 10 ms, isolation width of 2 m/z and activation q of 0.25. Characterisation of naturally occurring intact peptides of VGF was performed using Proteome Discoverer (PD) software (version 1.4, Thermo-Fisher) with the SEQUEST HT cluster search engine (University of Washington, licensed to Thermo Electron Corporation, San Jose, CA) against the target *rattus norvegicus* VGF sequence obtained from the Uniprot KB database. The database search parameters were N-terminal pyroglutamic acid residue and C-terminal amidation as dynamic modifications. The peptide mass tolerance was set to 10 ppm and the fragment ion mass tolerance was set to 0.02 Da. Peptides were filtered for high confidence and a minimum length of 6 amino acids; FDR settings were 0.01 (strict) and 0.05 (relaxed).

2.7 Brain staining

Rats were deeply anaesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused-fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.2–7.4. Brains were rapidly removed, washed overnight in PBS containing 7% sucrose and 0.01% NaN3, oriented in aluminium foil moulds in cryo-embedding medium (Cocco et al., 2003) and frozen in melting freon (cooled with liquid nitrogen). Coronal cryosections (10 µm) encompassing the entire SN and/or CPu obtained from the midbrain (from the section with AP≈-6.5 to the section with AP≈-4.5) were collected on poly-L-lysine-coated slides and stored in the vapour phase of a liquid nitrogen tank until use. IHC investigations were performed on 12 treated rats, with 6 animals from each group (fipronil and DMSO) chosen. The remaining rats (out of a total of 24 treated rats) were not chosen because their brains were clearly not properly perfused (brains still showed red vassels, thus IHC was not conducted) or were only partially perfused, making it difficult to detect positive labelling when performing IHC. IHC was carried out using our rabbit anti-VGF C-t antibody, (1:500), the sheep anti- TH (1:600;) and the two commercial VGF C-t antibodies, (# PA5-20523; against 17 aa at the C-t and PA5-63081

against 100 aa at the C-t); all diluted in PBS containing 30 ml/L normal donkey serum, 30 ml/L normal rat serum and 0.02 g/L NaN3. Sections were incubated overnight in a humidity chamber with the primary antibodies, while the appropriate species-specific donkey secondary antibodies conjugated with either Cy3 or Cy2 were used to detect primary antibody reactivity. Slides were coverslipped with PBS-glycerol (40%), observed and photographed using BX41 and BX51 fluorescence microscopes (Olympus, Milan, Italy) equipped with FujiS3 Pro digital cameras (Fujifilm, Milan, Italy). Routine controls included sequential substitution of each antibody with PBS, use of pre-immune or non-immune sera, and testing of each secondary antibody with its respective non-relevant primary antibody. For semi-quantitative determination of TH and VGF immunofluorescence signal, the FIJI image processing package based on ImageJ (NIH) was used. Briefly, 4 sections/animal/group/areas (SN or striatum) were selected, images were taken under standard exposure conditions with the S3 Pro digital camera (magnification: 4x) to cover the entire SN, including the pars compacta and reticulata. The RGB TIFF images were first converted to 8-bit greyscale, then the boundaries of the SN were manually traced by the user, and finally the background fluorescent signal was removed by a manual thresholding process. The mean SD of the optical density (OD) of binding of each TH and VGF antibody was then calculated and used for statistical analysis for each group of animals.

2.8 Statistical analysis

According to the 3Rs principles, we aim to minimize the number of animals used. To this scope, sample size calculations were performed before starting the experiments to ensure adequate experimental group numbers to be used in the study. Based on these calculations, it was expected that a number of 12 rats would be sufficient to detect significant differences between groups in the locomotor activity tests (t test, effect size d = 1.2, power (1-β) = 0.80, α = 0.05), while a number of 6 rats/group would be sufficient to detect significant differences between groups in the IHC assays (t test, effect size d = 2, power (1-β) = 0.80, α = 0.05). These calculations have been done based on prior studies using similar protocols (see for instance, Angioni et al., 2016; Bharatiya et al., 2020a,b; Sanna et al., 2021) and were carried out by using the software G*Power 3.1 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologieund-arbeitspsychologie/gpower). Behavioral data were expressed as mean ± SEM and analyzed by Student's t-test (in case of the analyses of 30 minutes total counts) or repeated measures two-way ANOVA (in case of the analyses of fractional 5 minutes counts) with the Treatment as a between-subject factor and the 5 minutes Time fractions as a within-subject repeated measure. When ANOVA detected significant effects of main factors or interactions post-hoc comparisons

were performed using Bonferroni's corrected pairwise contrasts. Before running the tests, normal distribution of data was ascertained by the Shapiro-Wilk test and homogeneity of variances was verified by the Bartlett test. The analyses were carried out with PRISM, Graph Pad 8 Software (San Diego, USA) with the significance level set at $p < 0.05$.

The OD collected from IHC experiments was statistically analyzed using Microsoft Excel StatistiXL software. For each experimental set, the normality of the data distribution was preliminarily checked using the goodness-of-fit test. The resulting p-values were > 0.05 in all cases; therefore, the following parametric tests were applied. Sample variances were remeasured using the F-test for equality of variance; therefore, individual or pooled variances were used for the two-tailed Student's t-test. P values < 0.05 were considered significant.

3. RESULTS

3.1 VGF fragments in the SN and striatum of untreated rats

Our VGF antibody detected a 75kDa band compatible with the pro-VGF using WB performed with brain samples (SN, CPu, accumbens and hypothalamus) each from 4 untreated rats (Fig. 2). The same band was also labelled using $PC12^{+}$, but not with $PC12^{-}$. These results are consistent with the top-down nano-RP-HPLC-high resolution ESI-MS/MS analysis done using untreated rats (n=2 of SN and 3 of CPu), which identified intact peptides (numbering seven in the SN and seventeen in the striatum), largely covering the VGF sequence. Importantly, among the fragments containing the C-t portion, one of 13 aa was characterized in the CPu (LQEQEELENYIEH peptide: $VGF_{599-611}$), whereas a longer peptide of 17 aa with the highest overlap with the immunogen used to generate our anti-VGF antibody (EQEELENYIEHVLLHRP: $VGF_{601-617}$) was identified in the SN. Although other bands smaller than pro-VGF (55, 35, 15 and 10 kDa) were also detected by WB (probably because they contain the C-t portion), these forms were not the same as those identified by HPLC analysis because of the different detection limits of the techniques. The complete list of peptides identified in both tissues is shown in Fig. 3 and reported in detail in the supplemental material (Tables S1 and S2). Comparing the two brain areas, the majority of peptides expressed in the SN are also found in the CPu, including the NAPP, whereas the CPu contains peptides that are exclusively expressed in this area, including the TLQP.

3.2 Immunostaining in treated rats

Immnuofluorescence was performed in DMS- and fipronil-treated rats and semi-quantitative statistical changes were investigated (n=6 each; Fig.4 a–l). Using SN sections of DMSO treated rats, in both injected and uninjected sides, TH staining was observed as expected, and our home-made VGF antibody labeled axon terminals possibly containing GAD and

Substance P, as previously reported (Cocco et al., 2020), but did not label dopaminergic perikarya. Using the SN sections for absorption experiments, almost complete prevention of labelling of our home-made VGF antibody was found at the high peptide concentration, while some brightness intensity was shown at the other peptide concentrations. The same labelling was also observed using the two commercial anti-VGF C-t antibodies raised against two C-t peptides partially overlapping our immunogen (Fig. 5). Using SN from unilaterally fipronil treated rats, the uninjected sides were comparable to both the DMSO -injected (Fig. 4 a, d) and -uninjected sides, with the TH antibody labeling cell bodies and dendrites as expected (Fig. 4, a), and our VGF C-t antibody staining neuronal terminals (but not cell bodies) within both the pars compacta and reticulata (Fig. 4, d). Instead, in the SN of the fipronil treated rats the injected sides (Fig. 4 b, e) revealed a decrease in TH immunostaining with fewer positive cell bodies and dendrites compared to the DMSO-injected (Fig. 4, b vs a), DMSO-uninjected and fipronil-uninjected sides. The decrease in TH immunostaining was more pronounced around the injection side, but remained reduced throughout the different sections examined. Correspondingly, both the intensity and brightness of the VGF C-t staining was reduced on the fipronil injected sides compared to DMSO -injected (Fig. 4 e *vs.* d, respectively) DMSO-uninjected and fipronil-uninjected sides and the reduction was more pronounced around the injection, but, as observed for TH, it remained reduced throughout the different sections examined. In the striatum of DMSO treated rats, in both sides VGF staining was observed in many areas of the brain, including the CPu, accumbens, and the cortex where cell bodies were labelled within the different layers, as well as the hippocampus and hypothalamus. Because neurons in the SN containing both GAD and substance P are known to be CPu projections, we focused on VGF labeling inside CPu. However, in DMSO treated rats (both sides), fipronil-injected and fipronil-uninjected sides, both VGF (Fig. 4 g and h, DMSO and fipronil injected sides, respectively) and TH (Fig. 4 j and k, DMSO and fipronil injected sides, respectively) staining were almost identical. VGF staining was distributed throughout the entire CPu and identified primarily in neuronal terminals and rarely in cell bodies, making it difficult to characterize a single location as the side of SN projections (other techniques should be used to properly describe it). To determine semi-quantitative alterations in immunofluorescence signals, we examined the OD for TH and VGF in both SN and CPu, comparing the fipronil to DMSO -injected sides. In the SN, OD for TH- (Fig. 4, c) and for VGF C-t- labelling (Fig. 4, f) showed a significant reduction after fipronil treatment ($p<0.05$). Instead, in the CPu, the TH (Fig. 4, i) and VGF (Fig. 4, l) staining did not differ when the entire CPu was selected in ImageJ to see if the overall OD changed in fipronil compared to DMSO -injected sides.

3.3 Locomotor activity after FPN injection into the SN

As reported in Fig. 6, rats unilaterally injected into the SN with a dose of 25 µg of fipronil displayed significant differences in locomotor activity 15 days after the injection compared to vehicle (i.e., DMSO) treated rats. In particular, fipronil-treated rats displayed a significant increase in horizontal activity; accordingly, two-way ANOVA of the counts referred to the 5 min fractions revealed a significant effect of treatment [$F(1, 22) = 9.48$, $p = 0.005$] (Fig. 6, a) and this difference was also confirmed by the t test performed on the 30 minutes total counts ($p = 0.005$) (Fig. 6, b). At variance, in the case of vertical activity, two-way ANOVA detected a significant treatment x time interaction [$F(5, 110) = 4.42$, $p = 0.001$] (Fig. 6, c) indicating slightly lower values in FPN-treated rats when compared to vehicle-treated ones. However, this difference was not more detectable when analyzing the 30 minutes total counts by the t test (Fig. 6, d). Finally, no significant differences between groups were observed in the center time, nor by the ANOVA on 5 minutes fractions neither by the t test on total counts (Fig.6, e, f).

4. DISCUSSION

The results of our studies showed that fipronil injection induced not only a reduction in TH, as previously reported (Park et al., 2016; Bharatiya et al., 2020a), but also in VGF staining, in parallel with changes in locomotor activity, that were detectable 15 days after the intranigral injection of the pesticide. As for our anti-VGF antibody, it has previously been used in SN (Cocco et al., 2020), but here we made a further effort to better characterise it using affinity purification, WB analysis (also using PC12 cells), and commercial antibodies against C-t sequences partially overlapping our immunogen and validated for their reactivity against pro-VGF. WB analysis confirmed that our VGF antibody recognised pro-VGF in the SN and striatum. These data are consistent with HPLC-MS which, although unable to recognise the 75kDa pro-VGF form, detected several fragments covering a large part of the VGF sequence. Among the VGF peptides, those containing C-t sequences partially overlapping with the immunogen used to generate our VGF antibody were identified in the striatum and SN. To the best of our knowledge, this is the first report of the identification of multiple naturally occurring VGF fragments in the SN using HPLC-MS, while some VGF fragments were previous found in the rat striatum (Karlsson et al., 2013) and striatum secretome (Bernay et al., 2009). Thanks to this finding, specific VGF peptides could be used to explore experimental approaches with specific therapeutic purposes using animal models. Furthermore, HPLC-MS, which is independent of antibody reactivity, confirmed the results

obtained by IHC and led to the hypothesis that the VGF changes in the SN under fipronil treatment are related to pro-VGF and/or its C-t truncated peptides.

In our previous investigation (Bharatiya et al., 2020a) we observed that the microinjection of 25 µg FPN into the SN induced 15 days later a decrease of about 40-50% in dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) tissue levels in the striatum, that was paralleled by a decrease to a similar extent of TH immunoreactivity in the SN. Here, although we confirm the changes observed in TH immunoreactivity in the SN, we were unable to detect significant TH immunoreactivity changes in the striatum. These contrasting results may be due to differences in the methods used; in particular, in our previous study we used high pressure liquid chromatography coupled to electrochemical detection (HPLC-ECD) that is more sensitive than the IHC used in the present investigation (on this regard, see also Yuan et al., 2005).

More surprising is the fact that VGF decreased in the SN but not in the striatum. In fact, VGF nerve terminals in the SN are probably projections from the striatum (Bolam and Smith, 1990; Cocco et al., 2020). However, striatal VGF is not only released in the SN but can also be transported into several afferent and efferent fibres, making it difficult to see differences if the change is confined to a small area. Furthermore, the mechanisms by which VGF is involved are not known, so a decrease in VGF within the nigrostriatal pathways could be associated with an increase in other(s) striatal pathways. Importantly, the present findings not only confirm, as in our previous study (Bharatiya et al., 2020a), that direct injection of FPN into the SN induces changes in locomotor activity (i.e., an increase in horizontal activity paralleled by a decrease in vertical activity) associated with dopaminergic degeneration, but also show that these changes can be associated with a decrease in VGF immunoreactivity within the SN. Although further studies are needed to elucidate the putative involvement and role of VGF in regulating pathways with a crucial role in locomotor activity and movement, our results are consistent with previous investigations linking VGF, dopamine and neurodegeneration. Indeed, a similar decrease in VGF was observed in rats treated with 6-hydroxydopamine, which was reversed by L-dopa administration (Cocco et al., 2010). Since the present work shows that environmental factors such as pesticides like fipronil can induce degeneration of dopaminergic neurons but also reduced release of VGF protein, it appears that VGF signalling can be directly regulated by dopamine function and is affected by low dopamine levels independently of the specific pathological processes involved in neurodegeneration, confirming its close relationship with dopaminergic neurotransmission, at least at the level of the nigrostriatal pathways.

However, given to the scarcity of studies on the subject, the involvement of VGF in nigrostriatal circuits is not yet obvious, and more focalized studies are required.
The behavioral alterations in locomotor activity induced by the fipronil microinjection in the SN deserve some additional comment. As recalled above, an increase in horizontal activity together with a slight decrease in the vertical one has been observed after fipronil treatment. There could be several reasons for this discrepancy in the direction of effects between horizontal and vertical activity. One of them relates to the fact that the two parameters quantify different aspects of motor behavior that involve partially different neural pathways. In fact, while horizontal activity (i.e., distance travelled in the horizontal plane) is usually considered an index of general motor activation and arousal and involves not only the activity of the dopaminergic nigrostriatal pathway but also that of the mesolimbic one (see for instance Sharp et al., 1987), vertical activity (expressed by the rearing behavior) is considered a more specific index of active exploration and has been specifically related to the nigrostriatal pathway and striatal dopamine function (see, for instance, Jicha and Salamone, 1991; Cousins et al., 1993). Thus, it is possible that the lesion induced in our rats, inducing a degeneration of the dopaminergic nigral neurons of approximately 60%, with a consequently relatively partial decrease of dopamine in the striatum can lead to a decrease in vertical activity (more sensitive to partial nigrostriatal damage) and, at the same time, to an imbalance in the activity of the pathways involved in the horizontal component of locomotor activity (nigrostriatal and mesolimbic pathways) that lead to an increase other than a decrease in horizontal activity. Support for this notion comes also from the observation that an increase in locomotor activity has been observed 6 days after treatment in rats with lesions of the nigrostriatal dopaminergic neurons induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine given at doses that produce a 50–60% decrease in striatal dopamine content (Ferro et al., 2005), although this difference was no more evident in the same rats after 18 days, pointing out the possibility that time-dependent alterations due to the time-course of the dopamine degeneration could be responsible of the different behavioral effects observed at different time-points in locomotor activity. To note, in the same study 6-OHDA did not induce any significant decreases in horizontal activity at both time-points, though a tendency to decrease rearing behavior was reported after 6 days. Noteworthy, the most part of the studies that observed significant reductions in locomotor activity after the lesion of the nigrostriatal pathway, were conducted inducing bilateral lesions able to provoke up to 80% (or more) dopamine decrease (Deumens et al., 2002) and often injecting the lesioning agent at the level of the medial forebrain bundle (MFB), thus also inducing the lesion of the dopaminergic

mesolimbic pathway. For this reason, this kind of lesions is usually considered as a model of end-stage parkinsonism (see Yuan et al., 2005) and can differ in several aspects from other models where the lesion is of lower entity, as in our case, and that could represent a model of early-stage parkinsonism. Further studies, in which the neurochemical and behavioral monitoring of rats injected with fipronil into the SN will last longer are needed to deeply investigate this possibility. Moreover, it should be also taken into account that at variance from other lesioning agents that are able to induce highly selective lesions of the dopamine system when injected into the SN or in the MFB (such as 6-OHDA), fipronil could also act on other neurotransmitter systems, inducing less selective lesions able to imbalance the activity of the basal ganglia at a more generalized level (see, for instance, Angioni et al., 2016). Finally, both the increase in the horizontal locomotor activity observed in the present study and the entity of the lesion induced in the TH immunoreactivity in the SN (by about 50%) closely resemble the findings reported and extensively discussed in our previous study (Bharatiya et al., 2020a), confirming the reproducibility of the model used. However, at variance from our previous study (Bharatiya et al., 2020a), we detected here a significant time x treatment interaction in the vertical activity data, indicating a slight, though significant, decrease in the vertical component of motor behavior, that did not reach significance (although present) in our previous study. However, this difference was not more significant when analysing the total scores of the 30 minutes test that leads to hypothesize that under the conditions used in this and our previous study this behavioral effect can represent a borderline finding that deserves further attention in next investigations.

5. CONCLUSION

Overall, further studies are needed to better understand the role of the pesticide fipronil as a predisposing agent for the development of PD or other neurodegenerative disorders. The fact that significant impairments in brain monoamine neurochemistry can be observed not only after direct injection of the pesticide into the SN (Bharatiya et al., 2020a), but also after chronic systemic exposure (Bharatiya et al., 2020b), deserves particular attention for the regulation and prevention of those environmental factors that in exposed humans may act as predisposing agents for the development of various neurodegenerative disorders, including PD.

Notably, fipronil can affect not only dopamine secretion but also the generation of pro-VGF and/or specifically VGF C-t truncated peptides in the nigrostriatal system, revealing a potential role in influencing locomotor activity. This could be valuable not only for

diagnostics, as previously proposed (Cocco et al., 2020), but also for experimental treatment methods based on the detected biological peptides.

FIGURE LEGENDS

Fig. 1 Timeline of the experimental procedures

Timeline of the experimental procedures was performed with the cohort of rats dedicated to the behavioral testing of locomotor activity. After a period of ten days of acclimation and daily handling, each rat underwent one habituation session in the motility apparatus that lasted for two hours in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Twenty-four hours later, the rats were tested in order to obtain basal values of locomotor activity and build the experimental groups. Then, the day after, DMSO or fipronil microinjection into the SN was performed. Finally, after a recovery period of 15 days, each rat was individually tested for locomotor activity in order to assess potential effects of the fipronil intranigral microinjection and, thereafter, rats were perfused, and brains collected for the *ex vivo* IHC assays.

Fig. 2 The molecular weight forms recognised by the VGF antibody

A band of approximately 70 kDa, compatible with pro-VGF, was recognised by our anti-VGF C-t antibody in samples of hypothalamus, substantia nigra, caudate-putamen (CPu), nucleus accumbens (n=4 each) and $PC12^{+}$, but not in $PC12^{-}$ (used as negative control); our antibody also recognised other smaller bands compatible with high cleavage of the pro-VGF at its C-terminus. PC12: pheochromocytoma cells, grown up with or without the nerve growth factor ($PC12^{+}$ or $PC12^{-}$, respectively).

Fig. 3 High-resolution MS/MS analysis

Naturally occurring peptides identified by high-resolution MS/MS analysis within the rat pro-VGF sequence in substantia nigra (from 2 untreated rats) and caudate-putamen (from 3 untreated rats). The blue and green arrows indicate peptides from the substantia nigra and caudate-putamen, respectively.

Fig. 4 Changes in treated rats

The TH and VGF antibodies were studied in DMSO-treated (unlesioned) rats *versus* the fipronil-(lesioned) rats (n=6 each group). In the subtantia nigra (a–f), the anti-TH antibody (a) labelled, as expected, several cell bodies and dendrites in the DMSO-treated (unlesioned) side, whereas in the fipronil-(lesioned) side (b) there was a significant reduction in the number of labelled neurons confirmed by a statistically significant decrease in optical density (OD) values (c, OD: 13.5 ± 3.3 *vs.* 47.4 ± 7.1; mean ± SEM; fipronil vs. DMSO sides). Our

anti-VGF antibody labelled axon terminals in the DMSO- treated (unlesioned) side (d), while in the fipronil-(lesioned) side (e) there was a significant decrease in brightness, confirmed by a statistically significant difference in OD values between the two sides (f, OD: 23.7 ± 7.7 *vs* 60.1 ± 7.6 mean ± SD, fipronil vs. DMSO sides). In the caudate-putamen (g–l), no differences were found for TH- or VGF- staining between the DMSO-treated (unlesioned) and fipronil-(lesioned) side (g and j *vs.* h and k, respectively), confirmed by the OD analysis (i, OD for TH:1331±249 and 1192±194 in the DMSO and fipronil lesioned sides, respectively; mean ± SD and l, OD for VGF: 2211±585 and 2005±283 in the DMSO and fipronil lesioned sides, respectively; mean ± SD). TH: tyrosine hydroxylase; scale bars: 50 um (a,b,d,e); 500 um (g,h,j,k).

Fig. 5 VGF Sequences

Sequences of the VGF immunogens used to generate the two commercial antibodies, composed of 100 and 17 aa (Ab_1 and Ab_2: # PA5-63081and PA5-20523, respectively), and our antibody (Ab_3); Ab=antibody; aa=ammino acids.

Fig. 6 Locomotor activity

Horizontal (a and b), vertical (c and d) and centre (e and f) spontaneous locomotor activity of the fipronil- (FPN, 25µg) and dimethyl sulfoxide- (DMSO,1 µL) treated rats. Rats were individually placed inside the apparatus and locomotor activity was recorded for 30 min (6 consecutive fractions of 5 min). On the left (a, c, e) are reported the values related to the individual 5 min recording fractions while on the right (b, d, f) are reported those related to the whole 30 min test. Values are mean ± SEM (n = 12 rats/group; t-test or two-way ANOVA followed by Bonferroni's corrected pairwise comparisons).

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

CCocco, FS contributions to concept/design, EM, GC, BN, MH, AM, BM, CContini, FS, AA, MRM, acquisition of data and data analysis/interpretation, CCocco and FS drafting and critical revision of the manuscript. All authors contributed to the revision of the manuscript and article approval.

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