Vagal innervation of the rat intestinal apparatus. A new quantitative approach on the basis of the tracer-fluorescence-intensity

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SUMMARY

In this methodological study, the effects of several parameters on the results of injections of a retrograde neurotracer into the gut were examined. To investigate the effects of tracer volume and the point of injection on the distribution, localisation and fluorescence of labelled cells in the dorsal motor nucleus of the vagus (DMN) after retrograde tracer transport, Fluoro-Gold was injected into the gut of male Wistar rats.

The tracer was injected into the wall of the intestine (cecum or anterior wall of the stomach corpus) of six rats; a control group of six rats received intraperitoneal application of the tracer. The tracer volume was varied. Neuronal labelling in the dorsal motor nucleus of the vagus was digitised under standardised conditions by means of software for measuring fluorescence and evaluated with data processing programs. The number of labelled cells, the location of labelled cells in the dorsal motor nucleus of the vagus, and the fluorescence of the individual labelled cells were examined. The control group was utilised to determine the degree of tracer diffusion into the peritoneal cavity after intramural tracer injection. After injection of a high tracer dose in a low concentrated solution into the cecum, a significant degree of tracer diffusion was detected. A reduction in the tracer dose resulted in a reduced number of labelled cells. In these cases, tracer diffusion can be excluded due to the small number of labelled cells. Injection of a small tracer dose into the anterior wall of the body of the stomach resulted in more labelled cells in comparison to the injection of an identical tracer dose into the cecum. In these cases no diffusion seemed to have occurred.

Evaluation of the fluorescence intensity of the labelled cells showed that the two cells with the highest fluorescence intensity of each individual rat were localised in the lateral area of the dorsal motor nucleus of the vagus after tracer injection in the cecum, and in the medial area of the left side of the dorsal motor nucleus of the vagus after tracer injection into the anterior wall of the stomach body. When compared to the results of other investigators, these results are confirmed. The use of a limited tracer volume and restricted survival periods seems to allow for correct vagus projection by this method. So far this correlation has not been examined for other tracers.

Key Words: Vagus – Axonal retrograde transport – Fluorescence tracer – Rat – Intestinal apparatus

INTRODUCTION

The introduction of retrograde tracer methods by Kristensson and Olsson (1971) was a great step forward in the investigation of neuronal connections between organs and their control systems within the CNS. The remaining problem areas in organ innervation are in most cases related to the high demand of tracer and in the application method used. By use of retrograde tracers the cell labelling results were improved. However, this method shows limited accuracy,

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particularly when even minor tracer redistribution to adjacent structures cannot be excluded (Fox and Powley, 1986, 1989). In order to prevent tracer diffusion, polymers were used to modify the diffusion properties (Griffin et al., 1979; Moskowitz et al., 1981) and adhesives to act as an artificial diffusion barrier (Yoshida et al., 1988, 1989; Sugitani et al., 1991; Baranowski et al., 1992; Hopkins et al., 1996). The evaluation of such methods is still problematic as it is impossible to recognise cell labelling caused by diffusion. Furthermore, additional substances and mechanical manipulation can influence the results (Moskowitz et al., 1981).

In the present study we examined under conventional, standardised conditions whether measuring the fluorescence intensity could provide us with information about the connection between the measured cell and the injection site[1]. The results may be influenced by the survival period and the amount of injected tracer (Fox and Powley, 1989). We therefore varied these parameters within strictly controlled ranges and examined the influence of these variations in the general fluorescence level as well as in the fluorescence intensity of strongly labelled cells.

The difficulties in examining the innervation of intra-abdominal organs are seen in a large number of divergent results. This is because it is necessary to use larger tracer injection amounts than is usual for labelling cerebral connections. This is likely to lead to an increase in tracer diffusion to neighbouring structures. Cells labelled by tracer diffusion may be found in regions not known to be connected with the primary injection site. The problem is exacerbated by the small distances between the different organs (Yu, 1980; Fox and Powley, 1986). As previously stated, it is not possible to recognise cells labelled by diffusion with 100 percent accuracy. Recent findings in the area of efferent motor innervation of the gut have shown an organotopic organisation of the DMN (Fox and Powley, 1985; Norgren and Smith, 1988; Okumura and Namiki, 1990; Altschuler et al., 1991; Berthoud et al., 1991; Altschuler et al., 1993, Zheng et al., 1999). This helped us to decide whether there was a direct connection between the injection site and the labelled cell in the DMN or not. An additional criterion was expected to be given by measuring the intensity of fluorescent label. The amount of tracer uptake by the neuron depends on the distance between the neuronal structure and the injection site. Additionally, the correlation between the experimental parameters (injection site and tracer amount) and the tracer diffusion causing a higher number of labelled cells with increasing fluorescence intensity has been not proven. Answering this question and optimising the tracer amount depending on the organ to examine could help to minimize tracer diffusion to neighbouring organs.

[1] To our knowledge, this technique has not been performed for such a purpose before.

METHODS

Twelve male Wistar rats (weight: 140–390 g) were used. All rats were injected with Fluoro-Gold (FG, Fluorochrome, Inc., Englewood, Colorado) in order to label vagal preganglionic neuron innervation in the injection site. The animals were subdivided into six groups (see Table 1).

Table 1. - Groups of animals studied.

Group	Group size	Injection type	Survival period in Days
IM 1	2	cecal injection (10 µl FG 2%)	5
IM 2	2	cecal injection (4 µl FG 2%)	4
IM 3	2	In the wall of the anterior body of the stomach (4 µl FG 2%)	4
IP 1	1	Intraperitoneal application (2 µl FG 2%)	3
IP 2	3	Intraperitoneal appli cation (2 µl FG 2%)	6
IP 3	2	Intraperitoneal appli cation (1,5 µl FG 5%)	3

A two and five percent FG solution was prepared from the crystalline FG powder, following the manufacturers instructions. Solutions of the same concentration have already been used by other investigators.

The animals were anaesthetised with chloral hydrate for injection procedures and for perfusion (400 mg/kg KG; 900 mg/kg KG), in addition, atropine was injected.

After a medial laparotomy under sterile conditions, no additional trauma occurred when preparing the organ selected for intramural injection (stomach or cecum). Using the immobile needle technique, the tracer was injected under microscopic control (Schroeder and Mestres, 1986, 1987). No tracer reflux occurred when tunnelling under the serosa for a long distance (10mm). The injection point was then cleaned with cotton swabs and the peritoneal cavity was washed with sterile saline solution to avoid contamination of other structures.

In all IP animals the tracer was applied intraperitonealy following a laparotomy to guarantee good tracer distribution and to exclude the risk of organ injection. In all groups, a single-shot antibiotic (Metronidazol 1.65 mg and Cefuroxim 2.47 mg in 1 ml solution, intraperi-

toneal application) was given before the wound was sutured.

For perfusion with formaldehyde following the protocol of Mesulam (1982), the rats were anaesthetised with an overdose of chloral hydrate. The brainstem was extracted and stored in the fixative solution (formaldehyde) at 4° C.

The brainstems were frozen and horizontal sections (20 µm thick) were cut using a cryostat and mounted on gelatine-coated slides. Following the tracer-manufacturers instructions, the slides were air dried, treated with Xylol and covered with DPX. For all procedures, the slices were protected from light (Baranowski et al., 1992).

To minimise errors in counting of DMN somata we counted only cells in every fifth section (i.e. a distance of 100 (m). The tissue was examined with a fluorescence microscope (Olympus) using light of 323 n.m. wavelength. Pictures (Ilford XP2) of the slices with labelled cells were taken using an integrated camera, thus avoiding the reduction in fluorescence intensity in labelled cells that can be caused by long-term fluorescence illumination during cell measurement.

Under standard conditions, the pictures were digitised with a video camera and the brightness of the labelled cells in a defined area was measured (Image pro plus, Media Cybernatics, Silverspring, MD, USA). For this purpose, a scale from 0 to 280 was used. Low values correspond to high fluorescence intensity and high values correspond to low fluorescence intensity. In order to avoid errors caused by development of negatives or variations in slice thickness, control measurements were carried out for each slice in areas having identical brightness values (central canal, fourth ventricle).

In addition to the fluorescence intensity, the location of the labelled cells in the medio-lateral and cranio-caudal direction was examined.

The measuring results were analysed statistically and the most important results were evaluated by means of diagrams.

For orientation in the rostro-caudal direction, the rostral pole of the postrema area (defined as the rostral-most section containing AP tissue that completely bridges the fourth ventricle) was used. This landmark was defined as Bregma -13,8 mm (Paxinos and Watson, 1986) and was used to calculate the rostral and caudal extent of neuronal labelling following injections into different regions of the gut.

Following the findings of a longitudinal columnar organisation within the DMN (Fox and Powley, 1985; Altschuler et al., 1991, 1993), we divided it into three equal parts with regard to medio-lateral cell location. We examined the influence of the injection site and tracer amount on cell position and fluorescence intensity of the labelled cells.

For the intraperitoneal tracer application we also examined whether an increased survival period (6 d) and/or a higher tracer amount might affect the labelling pattern (e.g. number of labelled cells) or the fluorescence intensity of the labelled cells.

For the intramural injections, we regarded a survival period of 4 to 5 days as necessary for the transport of a sufficient amount of tracer to the brainstem but safe regarding tracer diffusion to other intra-abdominal regions.

RESULTS

Labelled cells in the DMN were seen in all animals (Fig. 1). Nevertheless, there were differences in number, position and fluorescence intensity.

Intraperitoneal Application (IP)

In all animals with IP application we found an equal amount of labelled cells in corresponding sections of both sides of the DMN, with a maximum of cell counts in the rostral part of the nucleus. No region of the lateral extension was evidently devoid of labelled cells. Variations in labelling pattern could be found throughout the rostro-caudal region (Fig. 2a and Fig. 2b). There was no correlation between the tracer amount and the survival period with respect to the presence of labelled cells in the pole regions of the DMN. There was also no correlation between these experimental conditions and the extensive variation in the number of labelled cells (Fig. 3).

In all ip-animals we generally observed a weak fluorescent label (FS>200, Fig. 4) without an accumulation of stronger fluorescent cells. Only in one case (IP2-1) did we find a group of cells with stronger labelling. This group was situated in the left rostral region of the DMN, occupying the lateral two thirds of the nucleus.

The differences in labelling pattern could only be seen when measuring the fluorescence intensity.

Intramural injections (IM)

When we injected a larger tracer amount in the cecum (IM1), fluorescent cells were found throughout the DMN without a specific side or local preference. Most cells were located in the rostral part of the DMN (Fig. 5a).

Measuring the fluorescence intensity, we found values occupying a wide range of the fluorescence scale. The cells with high values (low fluorescence intensity) were located throughout the rostro-caudal and bilateral regions of the nucleus. The cells with low values (high fluorescence) were concentrated in the rostral and the middle part of the lateral third of the nucleus (Fig. 5b). These cells had the most intensive labelling found in all animals investigated (Fig. 4).

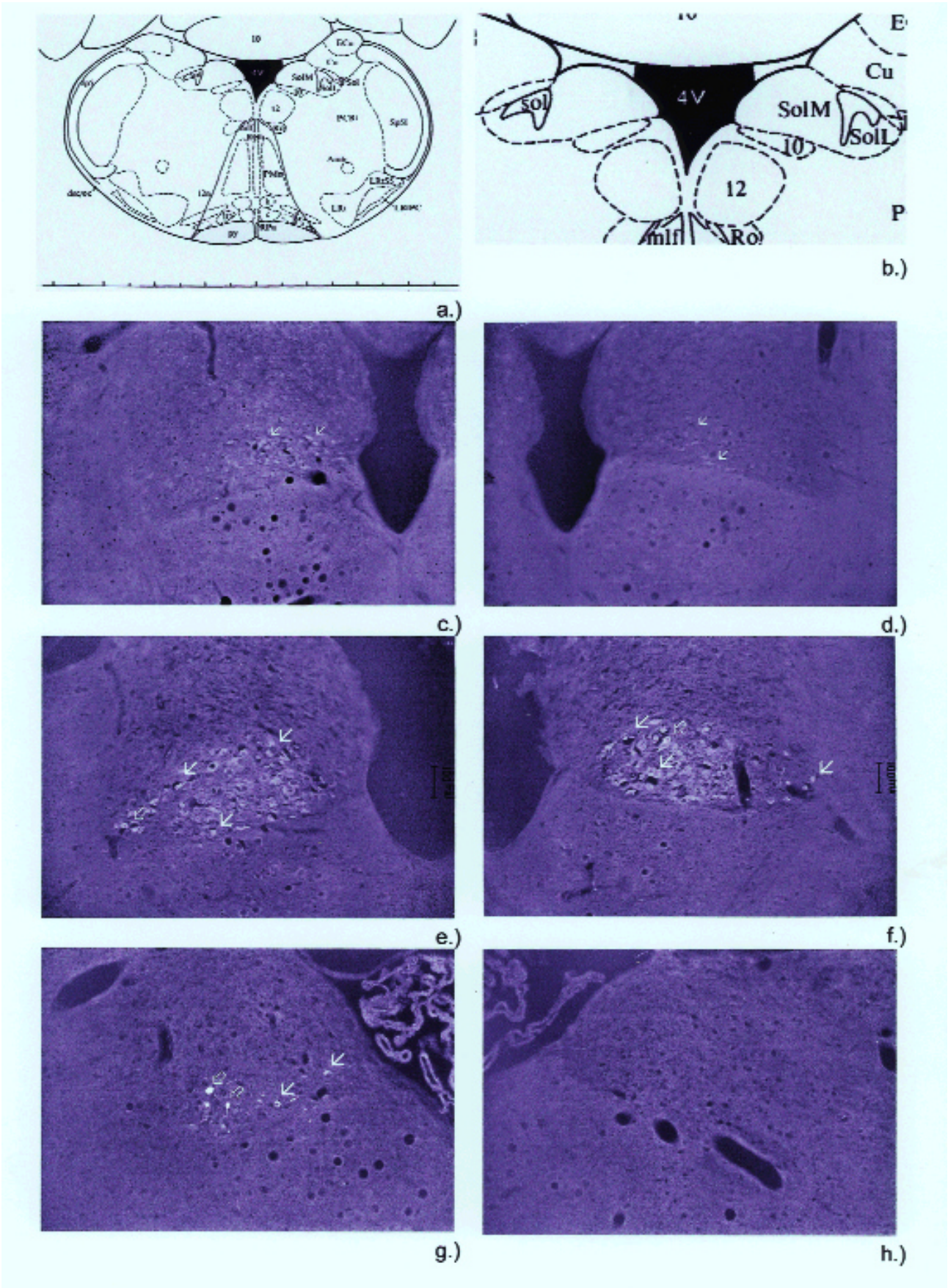

Figure 1.- In figures **a.)** and **b.)** schematic cross sections of different sizes through the brainstem and the DMN (10) at the level of Bregma –13.3 mm are shown. At this level the following pictures representing typical results of the different experiments were taken. Figures **c.)** and **d.)** represent typical fluorescence patterns following intraperitoneal application of a small amount of FG. Some of the weakly labelled cells are marked with small arrows.
A different labelling pattern could be seen in the following figures **e.)** and **f.)** of an animal receiving an injection of a large amount of tracer in the cecum. The strongest labelled cells are marked with - arrows.
Cells with fluorescence labelling were only found on the left side of the DMN after injections in the anterior wall of the stomach body [**g.)**, **h.)**]. Two very strongly and two strongly labelled cells are marked by arrows.

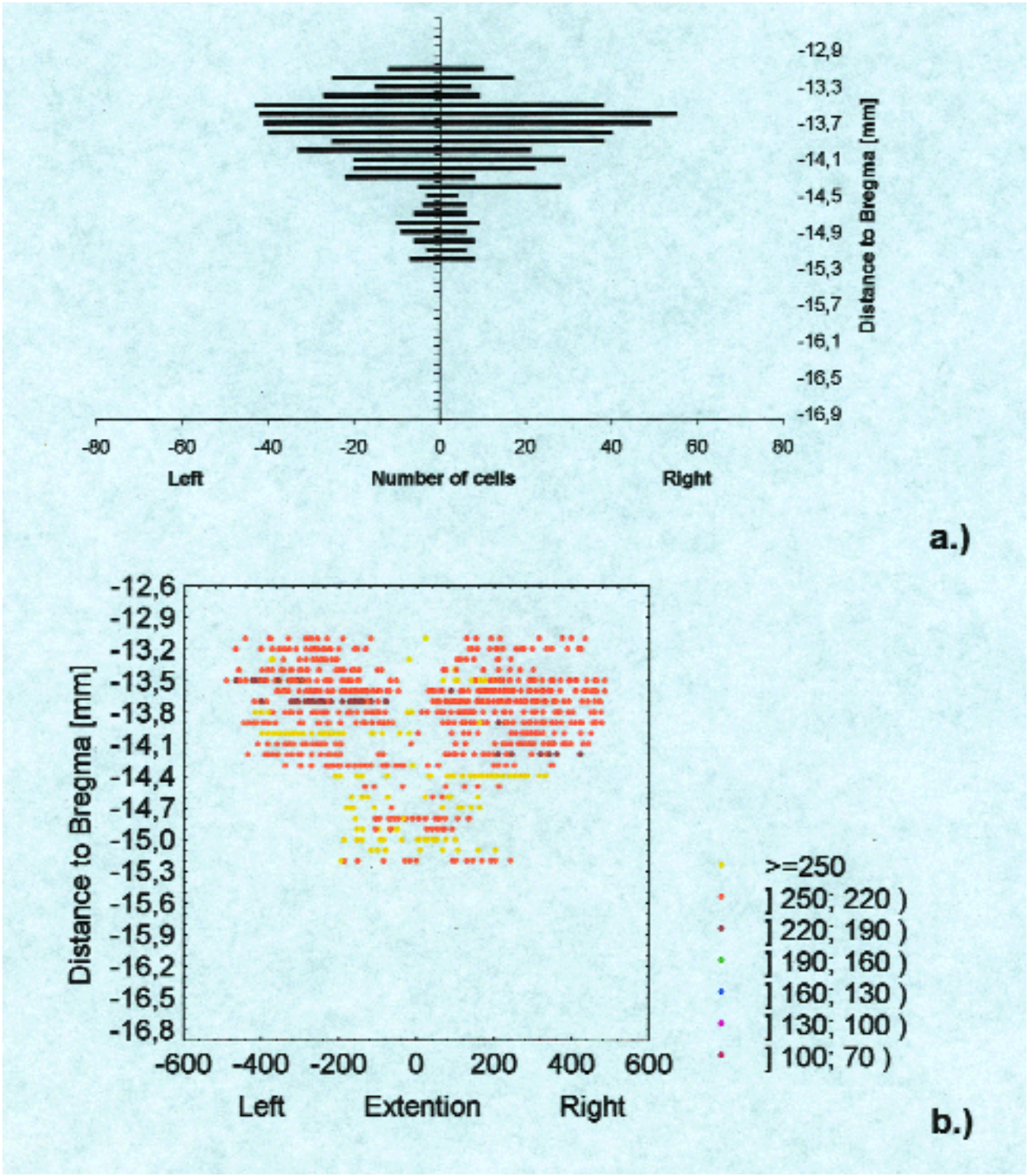

Figure 2.- **a.)** After intraperitoneal application of FG there was an equal number of cells labelled on both sides of the nucleus. **b.)** The fluorescence was generally weak (except IP2-1) and there was no accumulation of strongly labelled cells.

In the animals with a small amount of tracer injection in the cecum (IM2), only a small number of weakly fluorescent cells could be seen. The cells were located in the medial and lateral third of the rostral DMN. Those with stronger fluorescence intensity were found in the lateral third. When measuring the fluorescence intensity only values in the upper range of the scale were seen (Fig. 4).

When we injected the small tracer amount in the anterior wall of the stomach body (IM3), we found more cells with fluorescence labelling than after injection of the same amount in the cecum. However, there were fewer cells with fluorescence labelling than after the injection of the larger tracer amount in the cecum. Most labelled cells were located on the left side of the DMN, with a distribution in all three columns (Fig. 6a and b). In none of the other investigations did we find a preference for either side, as was the case within the DMN. In these animals, the cells with the most intense fluorescence

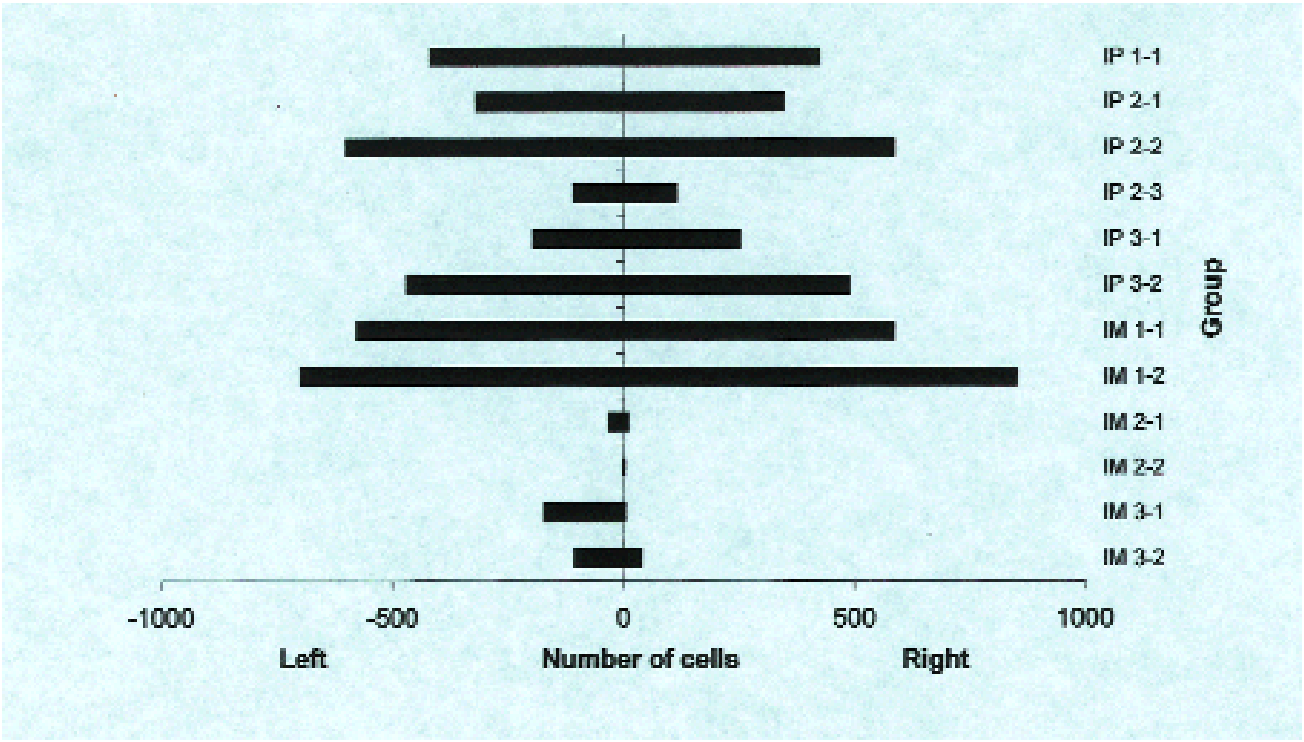

Figure 3.- Number of labelled cells in the different experiments.

labelling were located in the medial and middle third of the left side. The measured values of these cells did not reach the level of the strongly labelled cells found in the IM1 group. The weakly labelled cells were distributed throughout the medio-lateral and rostro-caudal regions of the left side of the nucleus.

Comparison of results

Figure 3 shows the number of labelled cells found in each animal. On comparing the number of labelled cells in the different groups, we observed that intraperitoneal application resulted in a greater number of labelled cells than intramural injection when the same tracer amount was used. Intramural injections of larger tracer quantities resulted in a similar number of labelled cells when compared to animals with a small IP application.

Only in the animals with tracer injection in the anterior wall of the body of the stomach did we find a predominance of labelled cells on left side of the DMN. We found no side preference in other animals, even with intramural injection.

Depending on the tracer amount and the location of intramural tracer injection, we found variations in the intensity of fluorescent labelling. The strongest labelled cells were seen in the animals that received an injection of a large tracer amount in the cecum. Injections in the anterior stomach body produced a labelling of comparable intensity. A weak fluorescence labelling followed injection of a small tracer amount into the cecum. With the exception of one animal (IP2-1), intraperitoneal applications led to weak fluorescence intensity (Fig. 4).

The position of the cells with the strongest fluorescence intensity was dependent upon the injection site. When the tracer was injected into the cecum, the cells with the strongest labelling were found in the lateral third of both sides of the nucleus. However, animals with injections in the anterior stomach wall resulted in the cells with the strongest fluorescence being located in the middle third of the nucleus. There was no correlation between the location of labelled cells in the rostro-caudal region and the injection site. In all animals, the cells with the strongest labelling were found in the rostral part of the DMN.

Discussion

Labelling after intraperitoneal tracer application

It is possible to label the vast majority, if not all, of the vagal preganglionic neurons projecting to the viscera when TB or FG are introduced into the peritoneal cavity (Sterner et al., 1985; Powley et al., 1987). The number and the fluorescence intensity of the labelled cells in the brainstem depend on the amount of tracer used (Sterner et al., 1985). For TB, the tracer doses necessary to reliably label all preganglionic neurons in the DMN (saturation dose) and the dose insufficient to label any cells in this nucleus (minimal dose, or lower threshold) are known. It is also known the lowest dose effective in labelling all neurons

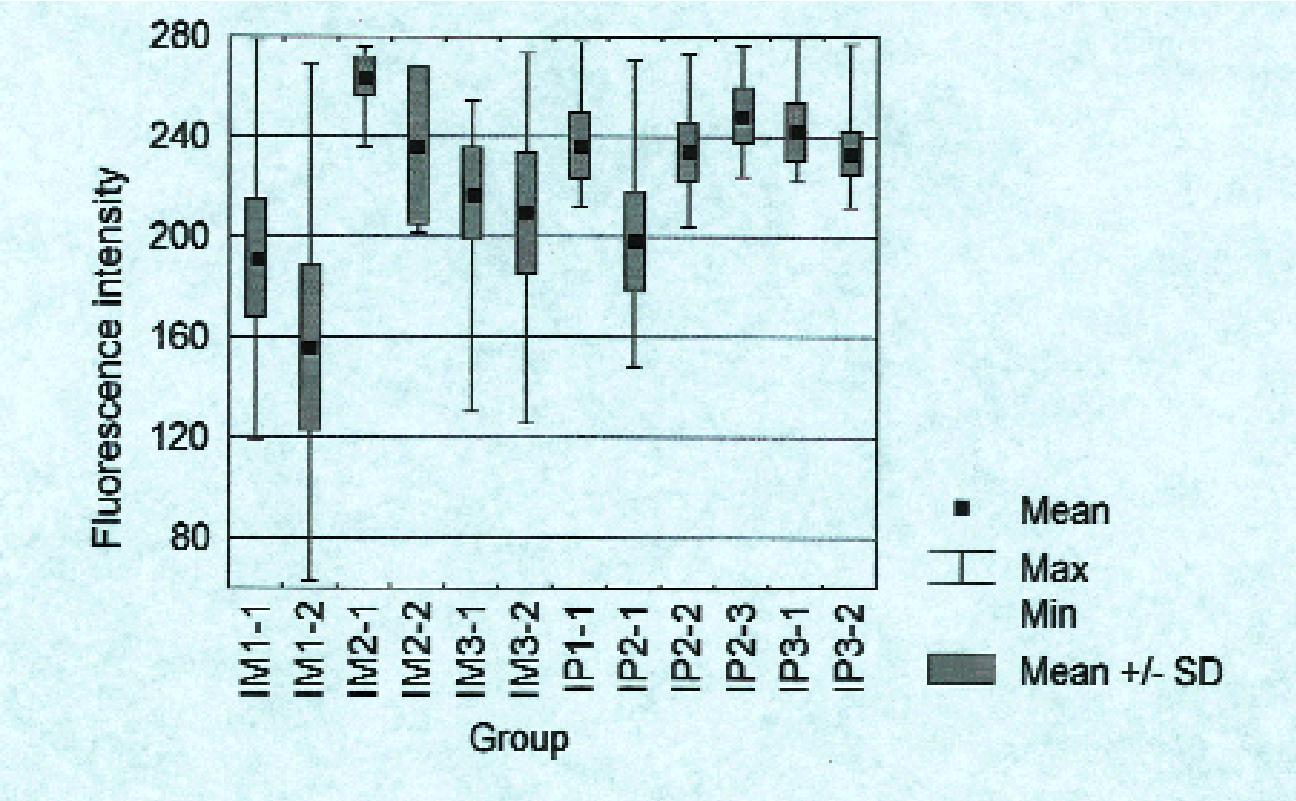

Figure 4.- Range of fluorescence intensity of cells measured in the different experiments.

that cannot be improved by a higher one (top threshold dose). The number of labelled cells correlates with the tracer dose in a nearly exponential function in the range from lower to top threshold dose. Based on these results, a dose 20 times higher than the top threshold dose is recommended for reliable labelling of all abdominal DMN projections. This dosage it has been used in order to control the effectiveness of a partial vagotomy (Powley et al., 1987). This dose value for FG is about 0,05 mg in rats (Sterner et al., 1985). In our IP animals, we applied an FG dose equal to the threshold dosage, and hence it was not surprising that there was no visible fluorescence in all cells of the DMN. Furthermore, there was considerable variance in the number of labelled cells and the range of rostro-caudal sections with fluorescence labelling. These findings could not be correlated with the variations in tracer amount or survival period.

In the medio-lateral direction, labelled cells were found on both sides, matching the results of Sterner et al. (1985). Often there was a lack of labelling in the pole regions of the nucleus. The nucleus has been reported to begin at Bregma -11,8 mm and extend to Bregma -16,4 mm (Coil and Norgren, 1979; Dennison et al., 1981a; Kalia and Sulivan, 1982, Sterner et al., 1985). If an absence of tracer transport can be excluded as the reason for the lack of labelling in the pole regions, a higher tracer uptake by the cells that were successfully labelled must be assumed. These cells are dominant in innervating the abdominal cavity.

In one of the animals with IP application, we found cells with a higher fluorescence level in the lateral pole of the left DMN. In comparison with the results of Fox and Powley (1985) and Altschuler et al. (1991, 1993), we concluded that the neurons of these cells must be a part of the accessory celiac branch of the vagus nerve. The most probable reason for this finding is a local tracer concentration caused by insufficient tracer distribution in the peritoneal cavity, followed by a higher tracer uptake by cells innervating this area. This “accidental” local labelling could only be detected when we measured the fluorescence intensity of the labelled cells. For this reason, we also expected more information about tracer diffusion from animals with intramural injections, when measuring the fluorescence intensity of all labelled cells. A differentiation between distribution errors with IP application causing local labelling and tracer spread in case of IM injection could not be expected.

Labelling after im injection

When FG was injected intramurally in our animals, we found a relationship between the number of labelled cells, their position in the nucleus and the fluorescence intensity of the cells with the tracer amount and the injection site (cecum or anterior wall of the stomach).

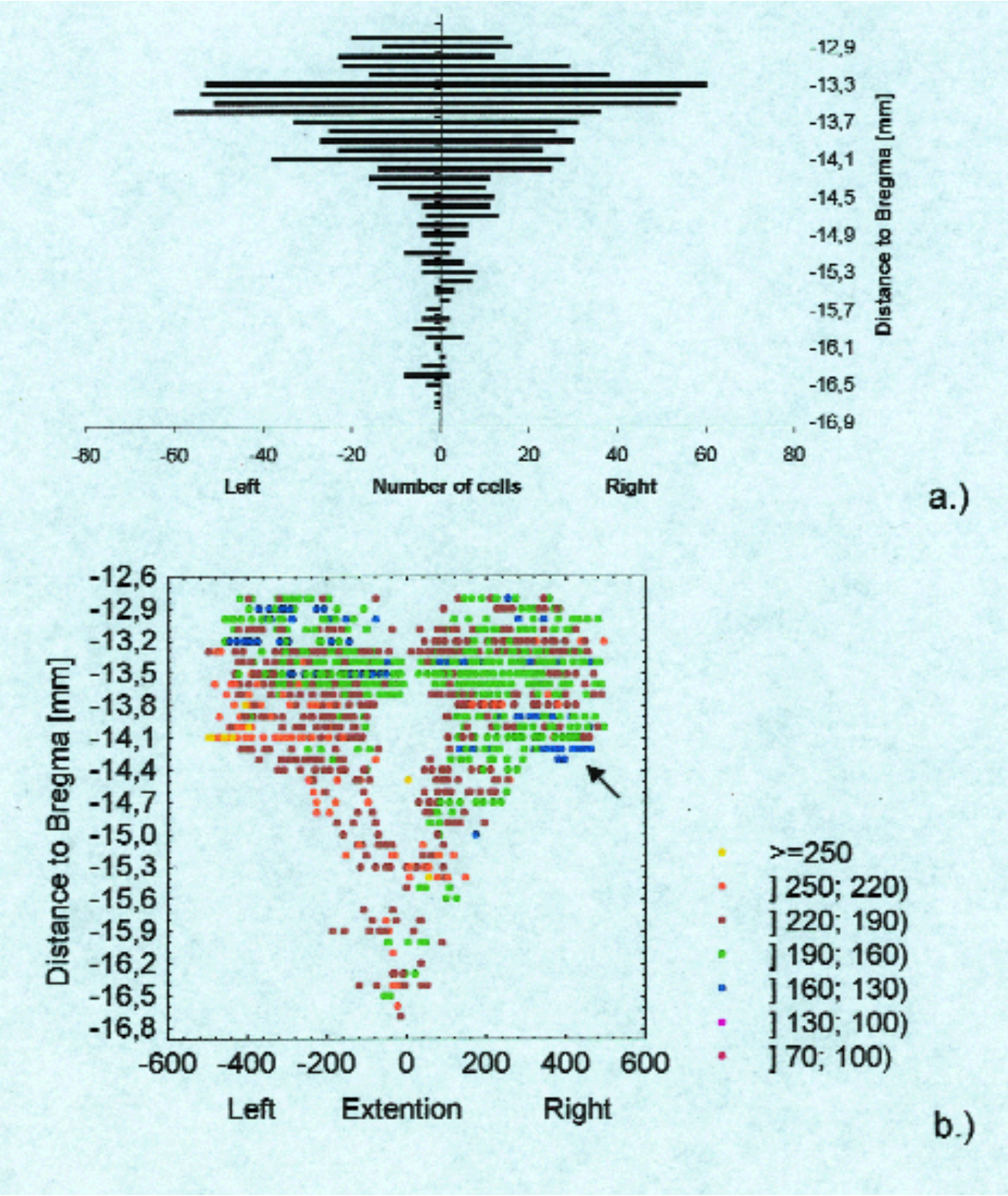

Figure 5.- **a.)** These figures show the result after injection of a large amount of tracer in the cecum (IM1). Nearly all cells of the DMN were labelled. **b.)** The group of cells with the strongest labelling is marked with an arrow. Compared with ip application there is a higher fluorescence intensity in most of the cells.

The greatest number of labelled cells was seen when a large tracer amount was injected in the cecum. The large number of labelled cells as well as the well-balanced distribution in all regions of the DMN –comparable to the results of IP administration (Sterner et. al., 1985)– can be explained by tracer diffusion.

There was a larger number of labelled cells in animals with tracer injected in the stomach than in animals with an equal amount of tracer injected in the cecum. In this case, it was not necessarily tracer diffusion that caused the higher number of cells, and the difference can be explained in terms of the well known closer

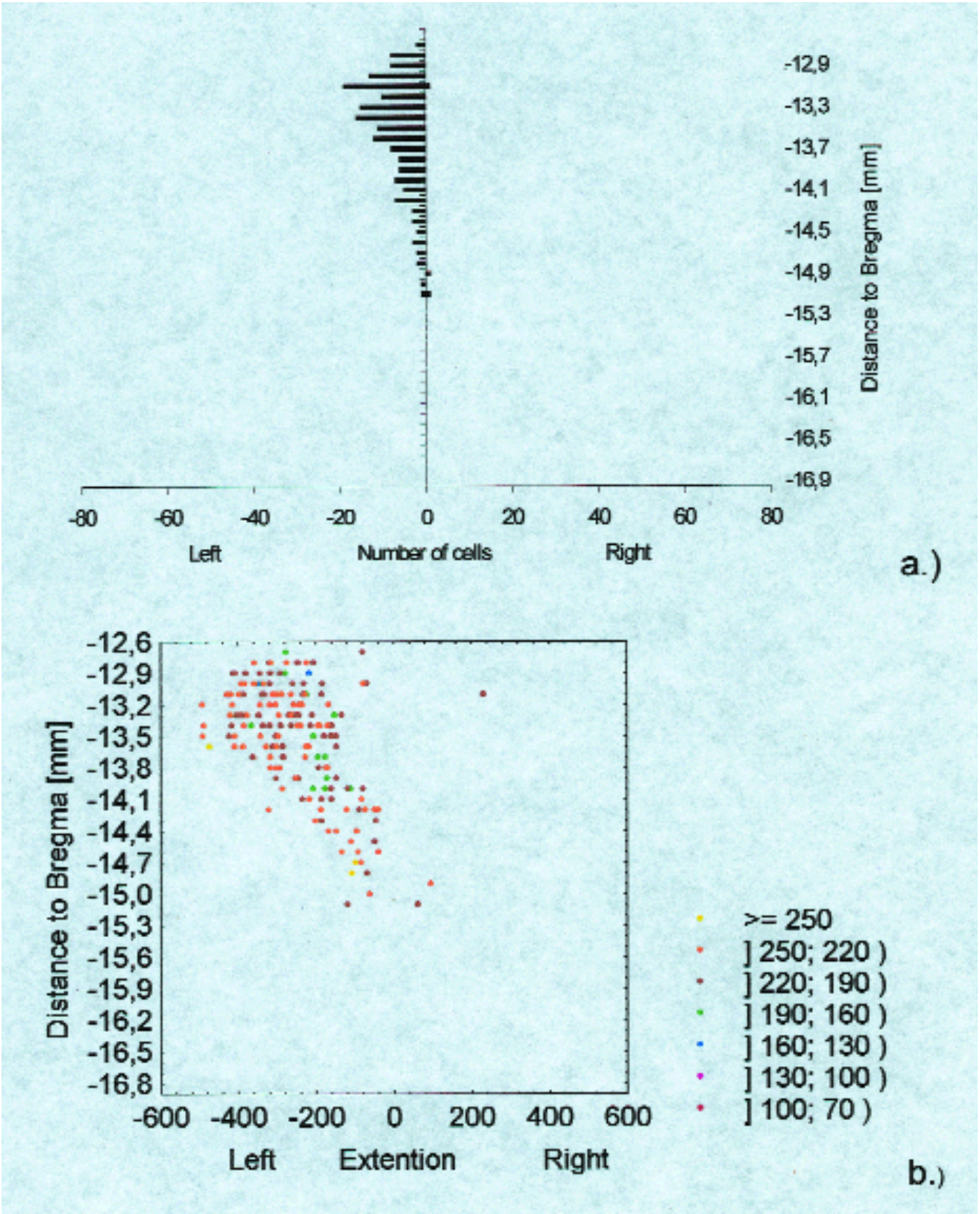

Figure 6.- **a.)** Injection of a small tracer amount in the anterior wall of the body of the stomach (IM3) produces labelled cells mostly located on the left side of the DMN. **b.)** The intensity of labelling was somewhere between the intensities found after IP application and cecal injection. Strong fluorescent cells arranged in clusters –as seen in figure 5b.)– were not detected in this group.

innervation of the stomach (Berthoud et al., 1991a). The preference for the left side of the DMN after im injections in the stomach wall is another point making tracer diffusion improbable. In the animals with im injection of a small tracer amount in the cecum, the number of labelled cells was very small. For this reason tracer diffusion in these animals could be reasonably excluded. The differences in the number of labelled cells dependent on the different

injection sites is in accordance with the findings of Sugitani et al. (1991). Nevertheless those authors failed to find a neuronal connection of the colon with the DMN, although this connection has been demonstrated several times (Berthoud et al., 1991b; Altschuler et al., 1993).

The cells with the strongest fluorescence labelling were also found in the animals with a higher tracer dose injected in the cecum. A slightly weaker fluorescence was produced when injecting a small tracer dose in the stomach wall. Only the injection of a small amount into the cecum afforded labelling values equal to those measured in the IP experiments. Thus, it follows that the number of labelled cells and the fluorescence intensity of the cells are correlated with the tracer dose and the injection site.

The position of the cells with the most intense fluorescence was independent of the tracer amount. Such cells were found in the lateral third of the nucleus when injections in the cecum were performed and in the medial two thirds following injections in the stomach. The longitudinal columnar organisation (Fox and Powley, 1985; Norgren and Smith, 1988), on the one hand, and the labelling pattern following injections in the anterior wall of the stomach (Yamamoto et al., 1977) or in the cecum (Satomi et al., 1978; Altschuler et al., 1991, 1993), on the other support our finding that these cells would be responsible for the innervation of the injection site.

Although FG is a tracer that diffuses easily, there are many advantages to using this tracer for our experiments. The tracer did not leak to neighbouring cells (Schmued, 1990), FG cumulated in the perikaryon (Schmued and Fallon, 1986), and produced strong fluorescence intensity in the case of a small tracer doses, making more easy the measurements. In addition, no histochemical processing is required, which could cause uncalculated changes in the fluorescence intensity, differing from rat to rat. The only drawback is a minimal fading of the fluorescence intensity when FG is illuminated for a long period (Schmued, 1990). Nevertheless, the measurements in our experiments were carried out from photographs to avoid the fading of cells that were only weakly labelled (Baranowski et al., 1992). Because FG is a strong fluorescent substance, the photos were taken with a shorter exposure time than with other tracers (Schmued and Fallon, 1986). The concentration of the injection solution was limited to 2 % to avoid a toxic effect on cells in the injection area or in the CNS. Concurrently, an increase in solution osmolarity, which could potentially disturb the integrity of the nerve membrane (Schmued et al., 1993), and an increase in serosa permeability at the injection site (Tesi and Forsmann, 1969) can be avoided.

In all animals, approved methods were used, such as microscopic control of injections, cleansing of the injection site and tunnelling of the serosa to minimise tracer diffusion. We refrained from penetrating the serosa several times to inject the tracer in smaller portions. We were thus able to minimise the trauma to the loose connective tissue in the gut. No tracer backflow out of the injection canal, reported by other investigators, was observed in our experiments.

In an attempt to reduce tracer diffusion we made changes in experimental designs proposed by different investigators. Yoshida et al. (1988) and Sugitani et al. (1991) used cyanoacrylate (loctite() to seal the wall of the gut. Although they were capable to show that FG was unable penetrate the barrier, tracer diffusion cannot be excluded by this method. Therefore, tracer uptake by blood vessels or fibres of passage still remains possible. Furthermore, an effect of cyanoacrylate on the results –e.g. blocking or reducing tracer uptake– cannot be excluded. This may be the reason why Sugitani et al. (1991) failed to observe any labelling in the DMN after injection of a high tracer dose in the sealed cecum, a connection reported by several other investigators.

To make safe injections of neurotracer in the gut, new knowledge of tracer properties is necessary. The correlation of tracer dose and effect with the number of labelled cells is unknown for most tracers. Powley et al. (1987) described the fluorescence intensity after application of different tracer doses intraperitonealy, but failed to measure fluorescence intensity.

In our investigations we found a correlation of the tracer dose necessary for visible labelling with the injection site. Whilst an injection of 0.04 mg FG in the cecum was required to achieve a visible level of labelling, a reduction in tracer amount would be possible when injecting in the stomach. When injecting a smaller tracer dose, the identification of neurones innervating the injection site should be easier. The tracer dose necessary for investigations in the innervation of the stomach cannot be concluded from our results.

The most intensive tracer uptake after injection occurs in cells innervating the injection site or the directly neighbouring area. When there is a correlation between fluorescence intensity and tracer uptake by labelled cells (as it is with FG), the fluorescence intensity may be a measure for the distance of the dendrites from the injection site. Even when only an intramural spread of tracer occurs, this will be diluted and nerve endings distant from the injection site will take up a smaller amount of tracer in lower concentrations. Accumulation of FG influences the tracer amount in the perikaryon, especially in the case of heavy arborisation of the nerve endings near

the injection site. Heavy arborisation is reported to exist in the stomach, whereas in the cecum the nerve endings are more rare.

Experiments measuring fluorescence intensity after injections of a retrograde tracer in the gut wall have not been reported previously (Berthoud and Powley, 1992). This is why today no standard procedure to avoid systematic mistakes when preparing the slices and measuring the fluorescence intensity has been available. The choice of a suitable retrograde neurotracer, as well as the definition of standard conditions for the whole process from tracer injection to measurement of fluorescence intensity, are fundamental to allow a comparison of results. Modern data-management systems and adequate measuring software mean that photographic documentation and manual positioning of the measuring square are not required. New software is now available for the automatic measurement of dark-light differences in defined regions of a picture. Thus, all pixels in a perikaryon are included. Visual control of the measured structure is still required since there are physiological intracellular substances emitting fluorescence –e.g. lipofuszin in older animals– that would lead to a misinterpretation of results.

In all experiments with retrograde tracer-transport described, the fluorescence intensity of the labelled cell was not thought to be important. The awkward procedure and the difficulties involved in comparing results without existing standards may be attribute to the lack of investigations into fluorescence intensity. Until the existence of a large data pool of comparable experiments, no classification of the results will be possible. Nevertheless, a relationship between the fluorescence intensity of a labelled cell and its role in innervating the injection site can be assumed. Although this is a suitable method for interpreting labelling patterns, it is not possible to sensitively detect tracer diffusion or to exclude it.

In conclusion, we found a correlation between the amount of tracer injected in the cecum and the number and the fluorescence intensity of labelled cells. Therefore, a reduced amount of FG will result in a more accurate labelling of the cells that innervate the injection site. For other injection sites, we did not look for this correlation although it should also exist. To prove this correlation, experiments with variations in tracer amount must be carried out. By measuring fluorescence intensity, it could be shown for all animals within one group (injection into the cecum as well as into the anterior stomach wall) that the labelled cells with the highest fluorescence were located in the same region of the nucleus. This was independent of amount of tracer and the total number of labelled cells. A relationship between the fluorescence intensity of a labelled cell and its role in innervating the injection site is highly probable.

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