Research Report

Chemogenetic inhibition of cells in the paramedian midbrain tegmentum increases locomotor activity in rats

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Abstract

Pronounced hyperactivity can be produced by lesions or pharmacological inhibition of cells in the median raphe nucleus (MR) located in the paramedian midbrain tegmentum. In the current study we examined whether a similar effect can be seen after chemogenetic inhibition of cells in this region using the DREADD (Designer Receptors Exclusively Activated by Designer Drugs) approach. We found that the DREADD ligand clozapine-N-oxide (CNO) increased locomotor activity in animals expressing the inhibitory DREADD hM4Di, but not those injected with a control virus in the MR. The effect was of rapid onset and short duration and persisted for at least four months after virus injections. Histological examination of the brains indicated that labeled fibers followed the known projection patterns of the MR to a variety of forebrain and midbrain structures. These findings confirm the role of the MR region in the control of locomotion and suggest that the DREADD technique may be a useful approach to the study of the functional architecture of this complex area. Methodological and interpretive aspects of DREADD studies are discussed.

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1. Introduction

The median raphe nucleus (MR), also known as the nucleus centralis superior, is a structure lying in the paramedian portion of the caudal mesencephalic tegmentum that appears to exert a remarkably powerful influence on a variety of behaviors. Especially pronounced effects are seen on measures of locomotor activity. Thus, marked increases in locomotion in a variety of settings are seen after electrolytic or excitotoxic lesions of the MR (Asin and Fibiger, 1983; Geyer et al., 1976; Lorens et al., 1971; Wirtshafter and Asin, 1982) or after inhibition of MR cells produced by local injections of GABA_A or GABA_B agonists or of excitatory amino acid antagonists (Wirtshafter et al., 1987, 1989, 1993). These effects are strikingly resistant to blockade by systemic administration of D_2 dopamine antagonists (Shim et al., 2014; Wirtshafter et al., 1988), suggesting that they are not secondary to alterations in dopamine release. In like fashion, increases in food intake...
can also be produced by intra-MR injections of GABA agonists and glutamate antagonists (Wirtshafter, 2000, 2011; Wirtshafter and Trifunovic, 1988). These effects on both activity and feeding are anatomically specific to the MR, and much smaller responses are seen with drug injections rostral, caudal, dorsal or lateral to the nucleus (Klitenick and Wirtshafter, 1988; Wirtshafter et al., 1989, 1993; Wirtshafter and Klitenick, 1990).

Although the MR is best known as a major source of serotonin projections to a number of forebrain sites including the hippocampus (Moore, 1981), the majority of MR cells utilize transmitters other than serotonin (Leger and Wiklund, 1981). For example, large numbers of neurons expressing various GABA and glutamate markers are found in the MR, and even serotonergic MR cells may colocalize other transmitters (Mintz and Scott, 2006; Mugnaini and Oertel, 1985). All MR projections studied to date contain a nonserotonergic component, although the relative proportions of serotonergic and nonserotonergic cells may well differ in various pathways (Aznar et al., 2004; Aznar and Knudsen, 2002; Szonyi et al., 2015). In line with these anatomical data, a substantial body of evidence indicates that serotonin plays, at most, a minor role in the effects produced by MR manipulations. For example, selective destruction of serotonergic cells does not reproduce the hyperactivity seen after nonselective lesions (Asin and Fibiger, 1983; Geyer et al., 1980; Lorenz, 1978) and intra-MR injections of serotonin autoreceptor agonists produce much smaller effects on locomotion than do injections of the GABA_A agonist muscimol, even at doses which produce similar effects on hippocampal serotonin release (Shim et al., 1997). These results all suggest an important behavioral function for transmitters other than serotonin in MR function.

Dissecting out the functional role of various chemically or connectionally defined populations of MR cells is a challenging task and one that would appear likely to be facilitated by the recently developed DREADD (Designer Receptors Exclusively Activated by Designer Drugs) technique (Urban and Roth, 2015; Wess et al., 2013). In one version of this approach, a virus is used to infect cells with a gene coding for a modified form of the inhibitory m4 acetylcholine receptor (hM4Di). This mutated receptor is insensitive to acetylcholine, but can instead be activated by the relatively inert agent clozapine-N-oxide (CNO). Thus, systemic administration of CNO will selectively inhibit neurons which express the DREADD construct. The effects of CNO can even be restricted to specific genetically defined populations of cells by injecting vectors for Cre dependent DREADDs into animals genetically modified to express Cre recombinase under the control of specific promoters (Shapiro et al., 2012). The hM4Di receptors are expressed not only in the cell body, but also in axon terminals, so it may even be possible to presynaptically inhibit transmitter release in terminal fields by local injections of CNO (Mahler et al., 2014).

Although the DREADD approach would appear to hold substantial promise for the study of the MR, there is currently no direct evidence that it will work in this system. The inhibitory DREADD method has been employed in a relatively small number of experiments in rats, as compared to mice, and appears to have obtained a reputation for being difficult to use successfully at the behavioral level in rats. In contrast, although a few behavioral studies of the MR have been conducted in mice (Martin and van den Buuse, 2008; Pezzato et al., 2015) the overwhelming majority of such experiments have been carried out in rats, a choice which is reasonable given the small size of this nucleus. In view of these considerations, we attempt in the current experiment to examine whether hM4Di mediated effects on cells in the paramedian tegmentum is able to produce alterations in locomotor activity similar to those seen after conventional pharmacological inhibition of MR cells. We examined the locomotor responses CNO both in rats transfected with non-Cre dependent hM4Di and in animals injected with a control virus which did not code for the DREADD construct. In order to examine how long responsiveness to CNO persisted following viral injections, we studied the response to CNO both in experiments beginning 20 days following viral injections and again four months later. We also examined whether CNO injections in DREADD-expressing animals would increase food intake, as do intra-MR injections of a variety of inhibitory drugs.

2. Results

2.1. Experiment 1. Effects of CNO in animals with DREADD virus injections into the MR

2.1.1. Effects on locomotor activity in rats with DREADD virus injections

Locomotor activity in response to injections of CNO at doses of 2.5 or 10 mg/kg, or its vehicle, was measured between 20 and 24 days following surgery and responses to the 10 mg/kg dose and vehicle were reassessed 140–142 days following surgery. Locomotor activity counts during the initial set of tests are shown in the upper panel of Fig. 1. Examination of the figure shows that CNO tended to produce a dose dependent increase in locomotion. This impression was supported by 2-way (dose × time) repeated measures ANOVA conducted on the 10–12 min long bins which followed drug treatments. This analysis indicated a significant effect of dose (F(2,10) = 10.06, p < 0.005) and post-hoc comparisons using the Fisher-LSD approach indicated that both doses of CNO increased overall activity with respect to vehicle, and that the overall response was significantly higher at the 10.0 mg/kg dose than the 2.5 mg/kg dose (p < 0.03 in all cases). Examination of Fig. 1 indicates that the response occurred with very short latency, being maximal within the first 12–24 min, and then decayed rapidly. The ANOVA indicated that the dose × time interaction was significant (F(18,90) = 2.37, p < 0.005, and post-hoc contrasts indicated that the dose effect was significant across the first 4 time bins (p < 0.053 for bin 1, p < 0.05 for bins 2–4), but not throughout the remainder of the session.

The lower panel of Fig. 1 shows the response to vehicle and 10 mg/kg CNO in the same animals tested approximately 120 days after the test described above. It can be seen that CNO again induced a short latency increase in locomotion which decayed to control levels over a relatively brief period. ANOVA indicated a significant effect of CNO (F(1,5) = 9.40, p < 0.05) and of the CNO × time interaction (F(9,45) = 2.60,
and post-hoc comparisons indicated activity was higher after CNO than vehicle for the first 3 time bins (36 min) following injections ($p < 0.05$), but not at later times. Comparison of the upper and lower panels indicates that the CNO response was substantially smaller at the delayed than at the original test.

2.1.2. Effects of CNO on food intake in rats with DREADD injections

In the 90 min. period following injections of vehicle, rats consumed a mean of $1.9 \pm 0.7$ g. Following injections of 10 mg/kg CNO, intake increased to $3.4 \pm 0.7$ g, a difference which is significant by a repeated measures ANOVA using a 1-tailed criterion ($F(1,6) = 4.94$, $p < 0.035$).

2.1.3. Viral injection sites

Injection sites in the MR were examined both for native fluorescence of the mCherry reporter, or following immunoprocessing with antibodies against mCherry. The latter method, especially using light field development with DAB as the chromogen, was especially useful in that it allowed for examination of the injections sites at low power where anatomical landmarks could be easily recognized. A representative injection site is shown in Fig. 2 where it can be seen that cells in the central portion of the MR expressed heavy mCherry-like-immunoreactivity (mCLI) which filled the region between the tectospinal tracts. (Some atlases would subdivide this region into the median raphe nucleus, sensu stricto, and a laterally situated paramedian raphe nucleus (Paxinos and Watson, 2007), whereas the more traditional view, which we adopt here, has been to consider this a single structure with median and paramedian subregions (Paxinos and Watson, 1982; Riley, 1943). The dorsoventral extent of the MR was involved in most animals. Some labeling of cells interstitial to the tectospinal tracts also occurred in all rats. Although the great majority of labeled cells were confined to the MR, some extraMR labeling was also observed in all subjects, although its location varied greatly between animals. In some subjects there was lateral spread into the ventral tegmental nuclei and the paramedian subregions (Paxinos and Watson, 1982; Riley, 1943). The dorsoventral extent of the MR was involved in most animals. Some labeling of cells interstitital to the tectospinal tracts also occurred in all rats. Although the great majority of labeled cells were confined to the MR, some extraMR labeling was also observed in all subjects, although its location varied greatly between animals. In some subjects there was lateral spread into the ventral tegmental nuclei and the paramedian reticular formation, in others some labeled cells were found dorsally in the dorsal raphe nucleus or ventrally in the nucleus reticularis tegmenti pontis or rostral to the MR in the caudal pole of the ventral tegmental area. The patterns of spread outside of
the MR itself were not obviously related to differences in CNO induced locomotor activity.

Even at the center of the injection site, viral labeling was found in only a subset of neurons. For example, Fig. 3 shows a typical section through the MR, taken at the site of maximal labeling, and processed with the neuronal marker NeuN. It can be seen that only about half of the neurons in this field expressed mCherry. Lower proportions were seen in regions farther from the injection site. No labeling was seen in cells not expressing NeuN.

2.1.4. Anterograde labeling
Anterogradely labeled axons were not apparent when viewing native mCherry, but could be easily visualized in immunocytochemically processed material. These fine-labeled fibers could be seen best in sections processed for fluorescence microscopy. A comprehensive study of anterograde labeling was not made, but selected levels were examined to determine the extent to which the observed projections matched those reported in previous studies of MR connectivity. Images captured from a representative case are shown in Fig. 4. Fibers descended from the injection site into the pontine raphe region and, more dorsally, into the caudal portion of the dorsal raphe and the central gray at the level of the rostral fourth ventricle. Strikingly, this projection almost entirely avoided the dorsal tegmental nucleus of Gudden (Fig. 4a). Rostrally, large numbers of fibers ascended through the ventral tegmental area into the lateral hypothalamus/medial forebrain bundle region (Fig. 4b). Labeling was much lighter in the ventromedial hypothalamus. Substantial labeling was also seen in the septal area and fibers could be followed into the hippocampal formation, where they formed a delicate plexus most pronounced in the dentate gyrus and CA1 fields (Fig. 4d). Heavy labeling was also seen in the lateral habenula, especially in its medial portion, whereas only a few fibers were seen in the medial habenula. Heavy labeling was also seen in the intralaminar and the midline thalamic nuclei, with much lighter staining present in the mediodorsal and paratenial nuclei (Fig. 4c). Scattered fibers were seen in the medial cortex, but very few fibers were apparent in the striatum or globus pallidus of most subjects.

2.2. Experiment 2. Effects of CNO in subjects with control virus injections
Following the completion of the experiments described above, locomotor activity was examined in a second group of subjects who received injections of a control virus not expressing the DREADD construct using methods otherwise identical to those employed in the previous study. As can be seen in Fig. 5, CNO injections appeared to be completely without effect on activity in these animals during either the tests begun two weeks following surgery, or those which took place 120 days later (p > 0.35 in all cases). Viral injection sites
in this study appeared similar to those in the first experiment. Anterograde labeling was not examined in these rats.

3. Discussion

The current findings demonstrate for the first time that injections of CNO in rats expressing hM4Di in cells in the paramedian tegmentum are able to significantly increase locomotor activity. CNO did not produce similar effects in subjects injected with a control virus, demonstrating that the observed responses were produced through an interaction with the DREADD construct, and did not simply reflect the weak pharmacological actions (Heiser et al., 2004; Schlicker, 1996) of CNO. Since many studies have now shown that CNO reduces activity of cells expressing the hM4Di construct (e.g., Nguyen et al., 2014; Parnaudeau et al., 2013; Robinson et al., 2014), it is overwhelming likely that these effects result from inhibition of the activity of paramedian tegmental cells. This conclusion is consistent with the fact that the behavioral effects observed here are similar to those seen in previous studies which employed lesioning (Asin et al., 1979; Asin and Fibiger, 1983; Geyer et al., 1976) and pharmacological inactivation (Wirtshafter et al., 1988, 1993; Wirtshafter and Klitenick, 1996) techniques to suppress the activity of cells in the MR. Histological examination revealed that substantial numbers of cells in the MR expressed the DREADD construct and the pattern of anterograde labeling we observed was extremely similar to that previously reported in studies of the MR which used non-viral tracers (Vertes et al., 1999; Vertes and Martin, 1988). (The morphology of our injection sites also appeared very similar to those examined in these anatomical studies.) Taken together, these results indicate that DREADD technology provides a promising approach to the study of the functional architecture of the MR region.

Certain aspects of our results highlight several important methodological issues which have not yet received extensive investigation. First, the behavioral effect of CNO persisted for at least four months following viral injections, a result consistent with the persistent cellular labeling seen at the end of the current study. In pilot studies we have sacrificed animals 2–3 weeks after injections and the observed labeling at that time did not obviously differ from that seen here. The locomotor response did seem to be reduced in magnitude when measured after a four month delay; this might reflect a partial loss of responsiveness with the passage of time, but might also be

*Fig. 4 – Illustrations of anterograde labeling following DREADD injections in the MR. In panel A, heavy mCherry-like-immunoreactivity (mCLI) can be seen in the central gray caudal to the MR, sparing the dorsal tegmental nucleus of Gudden. Panel B shows heavy labeling of fibers in the lateral hypothalamus; medial is to the left. Panel C shows staining in the medial and intralaminar nuclei with sparing of the paratenial nucleus, and Panel D shows labeling in the hippocampal formation. 3 = third ventricle; 4 = fourth ventricle; d = dorsal tegmental nucleus; dg = dentate gyrus; f = fornix column; m = medial longitudinal fasciculus; o = optic tract; p = paratenial nucleus.*
control virus injected with various doses of CNO in tests beginning either 20 days (upper panel) or 140 days (lower panel) following surgery. Each session consisted of a 60 min habituation period followed by a 120 min test period. Injections were given at the end of the habituation periods, 60 min after placement in the boxes. Insets show total activity over the 120 min period following injections. Large figures show activity across time in 12 min bins for the 60 min habituation period and the 120 min test period.

Fig. 5 – Locomotor activity counts for subjects treated with the control virus injected with various doses of CNO in tests beginning either 20 days (upper panel) or 140 days (lower panel) following surgery. Each session consisted of a 60 min habituation period followed by a 120 min test period. Injections were given at the end of the habituation periods, 60 min after placement in the boxes. Insets show total activity over the 120 min period following injections. Large figures show activity across time in 12 min bins for the 60 min habituation period and the 120 min test period.

related to a number of other factors such as the increased weight and age of the animals. Additional experiments would be required to determine whether or not the response is actually stable in magnitude for months after surgery, but the current findings do clearly demonstrate that the response does persist over this time period. Secondly, we observed that the response was dose dependent over the range of 2.5–10 mg/kg of CNO. In two pilot animals we examined a dose of 20 mg/kg which appeared to produce a still larger effect. The doses we used are similar to those applied in some previous rat studies (Mahler et al., 2014), but in others, effects have been observed at much lower doses (Boender et al., 2014; Ferguson et al., 2013). Studies in mice have also used a rather large range of doses (Wess et al., 2013). The reasons for these discrepancies are unclear, although the nature of the DREADD construct employed and the extent of cellular labeling may be important variables. These considerations do, however, stress the importance of examining a sufficient range of CNO doses before deciding that an effect is not present. Thirdly, the measurement of locomotor activity in well habituated animals provided an ideal situation for studying the time course of the effects of CNO, an obviously important topic which has received little study. We found that the response to CNO was of rapid onset and surprisingly short duration, peaking within 12–24 min after injection and rapidly decaying thereafter. These results are consistent with reports of rapid absorption and an extremely short half-life of CNO in mice (Guettier et al., 2009; Wess et al., 2013) and some behavioral studies in mice have observed similar brief durations of action (Nguyen et al., 2014). In contrast, some other studies have observed effects which persist for many hours following injection (Boender et al., 2014; Wess et al., 2013). The reasons for these marked discrepancies are not yet clear, but it is interesting that many of the studies reporting long duration effects have examined excitatory DREADDs. Obviously, blood levels are not the only determinant of effect duration, and processes such as desensitization are likely to play a role. At any account, the current findings emphasize the need for careful selection of testing times in conducting DREADD studies.

In addition to increasing activity, CNO injections in DREADD-expressing animals also tended to increase feeding. Although we observed a significant effect on locomotion, CNO-treated animals did not seem nearly as hyperactive as subjects we have observed in previous studies after intra-MR injections of GABA agonists or glutamate antagonists. The discrepancy in magnitude was even clearer in the case of feeding, where conventional pharmacological inhibition of MR cells produces very large effects on intake. It is likely that the brief time course of the CNO effects contributed to their small overall magnitude and it is also possible that larger effects would have been observed with a higher dose of CNO. It seems likely, however, that other factors are also involved. For example, many MR cells did not express the DREADD construct and thus would not have been directly affected by CNO; in contrast drugs like muscimol are likely to inhibit every cell within the nucleus. Additionally, it is plausible that not all cells which produce DREADDs express them in sufficient numbers to allow for a maximal effect of CNO. It is also possible that the maximal effect of CNO in DREADD-expressing neurons may be much less than that which can be obtained with conventional pharmacological approaches. All of these considerations suggest that considerable caution should be applied in the interpretation of negative results. Despite the many advantages of the DREADD technique, conventional pharmacological inhibition may have advantages with respect to determining the involvement of a brain structure in a particular behavioral function. In an attempt to minimize some of these difficulties we have examined several animals in
which we made repeated injections of the DREADD virus through chronically implanted MR cannulae. Strikingly, these animals showed larger behavioral responses, especially on feeding, than did those examined here, even though the extent of diffusion of the virus appeared similar to that in the current experiment. This method is likely to be worthy of further study as would be the effects of infusing different concentrations of the virus.

An important advantage of the DREADD approach is that it allows for a direct visualization of neurons transfected by the virus and thus sets an upper limit on the size of the affected region; in contrast it is very difficult to determine the extent to which pharmacological agents may spread from the injection site. Our present observation that alterations in activity did not appear to be associated with spread of the virus to particular distant structures supports the conclusion drawn from studies employing other techniques (Wirtshafter et al., 1989, 1993; Wirtshafter and Kliténick, 1990) that hyperactivity results primarily from an action within the MR or its immediate vicinity. Some labeled cells interstitial to the fibers of the tectospinal tract (predorsal bundle), which forms the lateral borders of the MR as here defined, were observed in all subjects; it is possible that modifications of the current approach using smaller injections or other viruses might be able to evaluate the involvement of this cell population. The DREADD approach also allows for confirmation that particular groups of cells incorporated the virus. In the current study the pattern of anterograde labeling indicated that MR neurons projecting to a variety of forebrain and brainstem sites incorporated DREADDs. The pattern of anterograde labeling observed was highly similar to that observed in most previous tracer studies, down to such details as the relative sparing of the dorsal tegmental nucleus and the medial habenula (Herkenham and Nauta, 1977; Morin and Meyer-Bernstein, 1998; Vertes et al., 1999; Vertes and Martin, 1998).

In contrast, a recent study using viral tracing techniques to examine the projections of serotonergic MR cells in mice (Muzerelle et al., 2014) noted heavy innervation of both the DTN and the medial habenula; the reasons for these discrepancies are uncertain, but might reflect species differences.) It should be noted that an action of CNO on terminals expressing the DREADD construct may have contributed to the observed behavioral effects.

In conclusion, the current experiments indicate that chemogenetic inhibition of cells in the paramedian tegmentum results in significant increases in locomotor activity, although the magnitude of this effect is smaller than that seen with conventional pharmacological inhibition of this region. DREADD is likely to provide a powerful tool for studying the functional architecture of the MR.

4. Experimental procedures

4.1. Subjects

Subjects in total were 13 adult male Sprague-Dawley rats obtained from Charles-Rivers (Chicago, IL), weighing about 350 g at the time of surgery, and individually housed on a 12:12 h light:dark cycle in plastic cages with food (Harlan 2018 rodent diet) and water available ad libitum. Two groups of rats were studied; seven animals were used in Experiment 1 to study the effects CNO in animals with DREADD virus injections and six subjects in Experiment 2, conducted several months later, which examined the effects of CNO in rats with injections of a control virus.

4.2. Viral vectors and drugs

The DREADD vector, AAV8-hSyn-hM4D(Gi)-mCherry and the control virus AAV8-hSyn-EGFP were obtained from the UNC Vector Core (Chapel Hill, NC). Clozapine-N-oxide (CNO, mol. wt. = 342.82) was obtained from the NIMH Chemical Synthesis and Drug Supply Program administered by RTI international (Research Triangle Park, NC). CNO was dissolved in dimethyl sulfoxide (DMSO) and then diluted with water to yield final concentrations of 2.5 or 10 mg/ml of CNO in 20% DMSO. The CNO was prepared immediately before use as it tended to precipitate if stored.

4.3. Surgery

Surgery was conducted using standard stereotaxic techniques and aseptic procedures under sodium pentobarbital anesthesia. Subjects also received s.c. injections of 5 mg/kg carprofen (Pfizer) prior to surgery. Stainless steel cannulae (28 g) were lowered into the MR at coordinates of AP: −7.8, H: −8.5, L: 0.0 (Paxinos and Watson, 2007) following retraction of the superior sagittal sinus (Wirtshafter et al., 1979) and 0.6 μl of the viral suspension (10⁹ copies/μl) was injected over a 10 min period. Animals were given two weeks to recover from surgery before the start of behavioral testing.

4.4. Locomotor activity

Locomotor activity was measured in a similar fashion in Experiments 1 and 2 using rectangular boxes measuring 78.5 × 21.5 × 21.5 cm³ (L × W × H) and divided into three compartments (Med-Associates, St. Albans, VT); transitions between compartments were detected by means of photocells and cumulated in 12 min time bins. On each test run, animals were placed in the center compartment of the box for a one hour habituation period, and removed briefly from the box and injected with CNO or vehicle and then replaced in the test apparatus for a further 2 h. Rats were given two weeks to recover from surgery and were then allowed to explore the apparatus in this fashion for five consecutive days with vehicle (20% DMSO) injections in order to habituate them to the environment. Animals then received three test sessions during which they received injections of either vehicle or CNO at doses of 2.5 or 10 mg/kg. (Thus, drug responses were examined on days 20, 22 and 24 following surgery.) Individual rats were tested in a counterbalanced order and individual tests were separated by at least one day on which animals were given a habituation session as described above. Approximately 120 days later, rats were again given three habituation sessions after which they were tested following injections of either vehicle or 10 mg/kg of CNO. The injections were counterbalanced and separated by 48 h and the subjects received a habituation run on the...
intervening day. One experimental animal received incorrect doses of CNO during the initial set of tests; his data are not included in the reported activity results.

4.5. Food intake

Food intake was assessed only in the DREADD expressing rats in Experiment 1. Immediately following the first set of locomotor activity tests, DREADD subjects received eight daily tests of food intake. In each of these they were removed from their home cages, and placed in hanging cages with grid floors containing a preweighed quantity of chow for a period of 90 min. On the first five days and on day 7, they were given vehicle (20% DMSO) injections prior to being placed in the test cages; on days 6 and 8 they were injected with either vehicle or 10 mg/kg CNO in a counterbalanced order. After each run, food intake, correcting for spillage, was measured.

4.6. Perfusion and histology

Following the completion of behavioral studies, animals were perfused under deep pentobarbital anesthesia with 10% formalin using a low/high pH protocol (Berod et al., 1981). After 2 h post-fixation brains were transferred for at least 24 h to a solution of 20% sucrose in phosphate buffered saline (PBS) at 4 °C. Cryostat sections were then taken at a thickness of 35 μm through the injection site and surrounding regions, and through selected levels in other parts of the brain. Some sections through the injection sites were examined directly for native mCherry or GFP fluorescence. Other sections through the injection site of DREADD expressing animals were processed for fluorescent detection of the neuron specific protein NeuN to allow investigation of the proportion of neurons which expressed the viral labels. Still other sections were processed using antibodies directed against mCherry and then developed for visualization using either fluorescent or light field techniques. These methods greatly facilitated the visualization of labeled axons and terminal fields and also made it possible to image the injection sites at low power. Light field techniques worked especially well for the injection site, whereas fluorescent approaches resulted in better contrast in terminal fields. For all immunocytochemical processing, sections were first thoroughly rinsed in PBS and then placed for 48 h at 4 °C in the primary antibodies prepared in PBS containing 0.2% triton X-100, 0.05% sodium azide and 2% of appropriate blocking serum. The primary antibodies we used were directed against either NeuN (Oncogene Research Products, La Jolla, CA, 1:20,000) or GFP or mCherry (Abcam, Cambridge, MA, both 1:15,000). Sections were then thoroughly rinsed in PBS after which they were incubated for 90 min in appropriate biotinylated secondary antibodies prepared in PBS containing 2% blocking serum. After further rinsing in PBS, sections were either developed for light field visualization using a Vector Elite ABC kit, or for fluorescent visualization by incubating them for 90 min in 1:300 dilution of Cy3-avidin, prepared in PBS. Sections were studied and imaged through a Leica epifluorescence microscope.

4.7. Statistics

Data were analyzed using standard repeated measures analysis of variance (ANOVA) techniques. When significant overall effects were obtained, there origin was examined using the Fisher LSD approach.

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