Iron Oxide Nanoparticle Delivery of Peptides to the Brain: Reversal of Anxiety during Drug Withdrawal

Nathan Vinzant 1, Jamie L. Scholl 1, Chia-Ming Wu 2, Trevor Kindle 2, Ranjit Koodali 2 and Gina L. Forster 1 *

1 Division of Basic Biomedical Sciences, Center for Brain and Behavior Research, Sanford School of Medicine, University of South Dakota, Vermillion, SD, United States, 2 Department of Chemistry, University of South Dakota, Vermillion, SD, United States

Targeting neuropeptide systems is important for future advancements in treatment of neurological and psychiatric illnesses. However, many of the peptides and their analogs do not cross the blood-brain barrier (BBB) efficiently. Nanoparticles such as iron oxide can cross the BBB, and here we describe a novel method for the conjugation of a peptide antisauvagine-30 (ASV-30) to iron oxide nanoparticles. Previous research has shown that direct infusion of ASV-30 into the brain reduces anxiety-like behavior in animal models via actions on corticotropin releasing factor type 2 (CRF2) receptors. Therefore, we tested whether iron oxide+ASV-30 complexes cross the BBB of rats and then determined whether iron oxide+ASV-30 nanoparticles are localized with CRF2-expressing neurons. Finally we tested the hypothesis that systemic infusion of iron oxide+ASV-30 can reduce anxiety-like behavior. First we describe the synthesis and demonstrate the stability of iron oxide-peptide nanoparticle complexes. Next, nanoparticles (87.7 µg/kg Fe2O3) with or without ASV-30 (200 µg/kg, ip) were injected into male rats 30 min prior to transcardial perfusion and brain fixation for immunohistochecmical analysis, or before testing on the elevated plus maze (EPM) in an amphetamine withdrawal model of anxiety. Systemically administered iron oxide+ASV-30 particles were present in the brain and associated with neurons, including those that express CRF2 receptors, but did not localize with the iron storage protein ferritin. Furthermore, systemic administration of ironoxide+ASV-30 reduced amphetamine withdrawal-induced anxiety without affecting locomotion, suggesting that the anxiolytic effects of ASV-30 were preserved and the bioavailability of ASV-30 was sufficient. The findings demonstrate a novel approach to peptide delivery across the BBB and provide insight as to the neural distribution and efficacy of this nanotechnology.

Keywords: iron oxide, nanoparticle, blood brain barrier, corticotropin-releasing factor, amphetamine, anxiety

INTRODUCTION

As our understanding of neuropeptide regulation of neuronal function increases, the need to effectively manipulate neuropeptide levels or receptor activity in the brain is becoming more necessary. Neuropeptidergic systems have been implicated in a variety of neuropsychiatric and neurological conditions including depression, anxiety, addiction, and neurodegenerative disorders
Forster et al., 2009) and increases anxiety-like behaviors in animal models of early life stress and amphetamine withdrawal (Vuong et al., 2010; Bledsoe et al., 2011; Reinbold et al., 2014). Similarly, CRF₂ receptors in the lateral septum appear to mediate anxiety-like behavior (Henry et al., 2006; Reinbold et al., 2014). Due to their effects on anxiety-like behaviors in animal models, CRF₂ receptors have been identified as a potential important target for anxiolytic drug development (Forster et al., 2012). However, like many neuropeptide receptor ligands, CRF₂ receptor ligands are peptides with low neurobioavailability if administered systemically.

**MATERIALS AND METHODS**

**Nanoparticle Synthesis**

Iron oxide nanoparticles (Fe₂O₃) synthesis was modified from Borcherding et al. (2014) and involved refluxing 8.7 mmol FeCl₃ solution containing 40 mL methanol and 5 mL water at 80°C for 72 h with drop-wise addition of 17.4 mmol NaOH solution containing 30 mL methanol. Iron oxide precipitate formed in the methanol solution, was washed three times using 14 mL of 50% v/v methanol-acetone and dried in an air oven for overnight at 80°C. Power X-ray diffractometry was used to identify the phase (Fe₂O₃) and purity (no other oxide phases were detected) of the resultant nanoparticles. Nanoparticles were characterized by transmission electron microscopy to estimate the particle size and size distribution, demonstrating that the resulting nanoparticles were ~5 ± 1 nm in diameter. Nanoparticles were then conjugated with 3-aminopropyltriethoxysilane (APTES) by refluxing the Fe₂O₃ nanoparticles with APTES at a 1:4 molar ratio in methanol for 48 h. The resultant powder was collected and washed with a mixture of acetone and methanol in a 1:1 volume ratio solution. The APTES-coated nanoparticles were dried overnight at 80°C and characterized using Fourier-Transform Infrared Spectroscopy (FT-IR) and Thermogravimetric Analysis (TGA) to confirm the presence of APTES and to estimate the amount of APTES groups. APTES functionalization was performed in order to effectively couple fluorescein isothiocyanate (FITC). 5 mg of APTES-coated iron oxide nanoparticles were dissolved into 0.5 mL, 1 × 10⁻⁵ molar
FITC in artificial cerebrospinal fluid (aCSF). The resulting solution was then sonicated for 10 min. After sonication, the powder was collected and washed in aCSF three times. The resultant Fe$_2$O$_3$ functionalized nanoparticles were monitored for stability over 48 h using UV-Vis spectrophotometry, and compared to UV-Vis spectrum of a $1 \times 10^{-4}$ molar Fe$^{3+}$ standard solution.

Antisauvagine-30 (ASV-30; Thermo Fisher Scientific, Hampton, NH) was attached to the resultant Fe$_2$O$_3$ nanoparticles by mixing APTES-coated Fe$_2$O$_3$ nanoparticles with ASV-30 in a 10:1 molar ratio in a vehicle solution comprised of 10% ethanol and 90% aCSF by volume. ASV-30 has multiple carboxyl (C=O) groups that bind to the amine (-NH$_2$) groups of APTES through covalent attachment and form amide bonds. In addition, hydrogen bonding and ionic interactions are also possible between ASV-30 and APTES-Fe$_2$O$_3$ nanoparticles. The solution was monitored hourly by FT-IR for up to 5 h to confirm the successful attachment of ASV-30 to the APTES-coating of the iron oxide nanoparticles. Finally, the stability of the APTES-coated nanoparticles with ASV-30 was assessed by FT-IR spectroscopy in aCSF.

**Animals**

Male Sprague-Dawley rats were bred within the in-house Animal Resource Center at the University of South Dakota. Adult (8–12 weeks old) rats were pair-housed in the same room under a 12 h reversed light cycle, with the dark period running from 10:00 to 22:00. Animals had free access to food and water, and cages were changed twice per week. All animal procedures were approved and performed in accordance with the University of South Dakota institutional animal care and use committee.

**Distribution of Systemically Administered Nanoparticles in the Brain**

Iron oxide nanoparticles are known to cross the BBB (Silva, 2008; Minchin and Martin, 2010; Yang, 2010), but it is not known if the conjugation of peptide to the nanoparticles will interfere with their transport kinetics. To address this, male adult rats ($n = 18$) were injected (ip.) with a APTES-coated Fe$_2$O$_3$ nanoparticle solution (87.7 $\mu$g/kg Fe$_2$O$_3$) tagged with FITC, or without ASV-30 (200 $\mu$g/kg; 100-fold greater than intracerebroventricular infusion; Reinbold et al., 2014). No adverse reactions were observed as a result of this injection. Thirty minutes after injection, rats were anesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with 150 mL 0.05 M phosphate-buffered saline (PBS) followed by 250 mL 4% paraformaldehyde (Meyer et al., 2012). Brains were collected and stored in 4% paraformaldehyde at 4°C for 20 h, followed by a series of two PBS washes for 24 h each with gentle agitation at 4°C. Brains were cryoprotected in a 30% sucrose solution followed by storage at −80°C. 40 µm coronal sections were taken using a freezing microtome.

Separate series of 40 µm sections were processed and imaged for either nanoparticle (FITC) with the neuronal marker NeuN, or nanoparticle and CRF$_2$ receptors with NeuN, or nanoparticle and ferritin with NeuN. Sections were washed three times using 0.04% goat serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 5 min at room temperature and blocked in 2% goat serum in PBS to reduce non-specific antibody binding. Sections were then incubated in either 1:500 rabbit anti-rat ferritin antibody (GenWay Biotech, San Diego, CA, USA; GWF-792B12) or 1:200 rabbit anti-CRF$_2$ receptor antibody (Novus Biologicals, Littleton, CO, USA; NB1-00768) with 1:750 mouse anti-NeuN antibody (Millipore Corporation, Temecula, CA, USA; MAB377; Barr et al., 2010) for 19 h at 4°C followed by 1 h at room temperature with gentle agitation on a shaker plate. Sections were then washed three times in 0.04% goat serum before being incubated in 1:200 goat anti-mouse Cy3 (Jackson ImmunoResearch; 115-165-003) or 1:200 goat anti-rabbit Cy3 (Jackson ImmunoResearch; 111-165-003) and 1:200 goat anti-mouse AMCA (Jackson ImmunoResearch; 115-155-003) secondary antibodies with gentle agitation for 2 h at room temperature. Sections then underwent three final washes in 0.04% goat serum in PBS and were mounted on subbed slides and cover slipped using Prolong Diamond Antifade Mountant (Life Technologies, Carlsbad, CA, USA). Sections from the lateral septum and dRN were then imaged using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus America, NY, USA) at 60x oil immersion. A Kalman scanning filter was used to exclude background noise not caused by specific antibody binding.

**Anxiolytic Effects of Iron Oxide+ASV-30 Nanoparticles**

All behavioral testing was conducted in the dark phase of the light cycle and were performed in a dark room illuminated by red lighting. To identify any potential effect of iron oxide nanoparticle administration on behavior in the elevated plus maze (EPM), rats were injected (ip.) with either unconjugated APTES-coated nanoparticles (87.7 $\mu$g/kg Fe$_2$O$_3$) in CSF or CSF alone, ($n = 11$ /group). Thirty minutes later, rats were placed in the center of the EPM (Noldus Information Technology, Leesburg, VA) and allowed to explore freely for 5 min, with their behavior recorded using an overhead camera via Mediacruise V.2.24 (Canopus Co. Ltd., Kobe, Japan). Following the test, rats were returned to their home cage and monitored for 3 days to identify any health effects of the nanoparticle injection, of which none were observed. Total distance moved and total time spent in open and closed arms of the EPM were scored by Ethovision XT 5.1 (Noldus Information Technology).

To determine anxiolytic effects of ASV-30 delivered as nanoparticle cargo, we utilized a well-established model of anxiety during amphetamine withdrawal (Barr et al., 2010; Vuong et al., 2010; Tu et al., 2014). A separate group of rats were pretreated with either saline or amphetamine (2.5 mg/kg, ip.) for 2 weeks and then allowed to undergo 2 weeks of withdrawal. Animals were then injected with either unconjugated APTES-coated Fe$_2$O$_3$ nanoparticles in aCSF (87.7 $\mu$g/kg Fe$_2$O$_3$, ip) or APTES-coated Fe$_2$O$_3$ nanoparticles+ASV-30 (87.7 $\mu$g/kg Fe$_2$O$_3$ and 200 $\mu$g/kg ASV-30, ip) 30 min prior to testing ($n = 13$ per group). Rats were tested on the EPM as described above.
Statistical Analysis
Data collected from the first behavioral study comparing nanoparticle administration to aCSF administration was analyzed using a one-way ANOVA. Data collected in the second behavioral study involving amphetamine withdrawal were analyzed using a two-way ANOVA test with factors of pretreatment and treatment. Significant main effects or interactions were followed by a Student-Newman-Keuls post-hoc test for multiple comparisons. All data were analyzed using SigmaPlot 11.0.

RESULTS
Characterization of Nanoparticle Complexes
The UV-vis spectrum of $1 \times 10^{-4}$ M Fe$^{3+}$ ions in solution showed two strong peaks at 275 and 209 nm (Figure 1A) that were absent in a suspension containing Fe$_2$O$_3$ nanoparticles conjugated to FITC incubated aCSF for 48 h (Figure 1B). This suggests that Fe$_2$O$_3$ nanoparticles do not dissociate into ionic iron (Fe$^{3+}$ ions) in vivo within 48 h of exposure to physiological conditions and this result is indicative of the stability of the nanoparticles prepared in this study. After ASV-30 was incubated with APTES-coated Fe$_2$O$_3$ nanoparticles for up to 5 h, a 50% reduction in the peak at 1,434 cm$^{-1}$ (symmetric vibration of COO$^-$ groups) was observed (Figure 2A), demonstrating that the COO$^-$ groups of the ASV-30 molecules had reacted with the amine groups of the APTES coating to form new amide groups on the iron oxide surface. Concomitantly, an increase in the intensity of the band at 1,537 cm$^{-1}$ (due to secondary N-H vibrations) was also observed, and this suggests the formation of new amide bonds as well. In order to understand the stability of the ASV-30 complex conjugated to the APTES-coated Fe$_2$O$_3$ nanoparticles, FT-IR spectroscopic studies were conducted. The FT-IR spectra of an APTES-coated Fe$_2$O$_3$ nanoparticles with ASV-30 complex and subsequently washed with aCSF showed characteristic peaks indicating that they are fairly stable in physiological aCSF.

Distribution of Systemically Administered Nanoparticles in the Brain
As illustrated by Figure 3, APTES-coated Fe$_2$O$_3$ nanoparticles conjugated to ASV-30 effectively crossed the BBB. No apparent differences in the distribution of nanoparticles were observed between tissue obtained from rats treated with iron oxide compared to iron oxide+ASV-30 in regions of the brain associated with anxiety states, including the dRN and lateral septum (Figure 3). Iron oxide nanoparticles were widely distributed throughout the brain and were not found in greater concentrations in any particular region qualitatively compared to another. Furthermore, iron oxide nanoparticles+ASV-30 were localized with CRF$_2$ receptor expressing neurons in the dRN and lateral septum (Figure 4). In contrast, iron oxide nanoparticles+ASV-30 showed little localization with ferritin in the dRN or lateral septum (Figure 5). Ferritin labeling was restricted to non-neuronal structures, likely glial cells (Figure 5, Moos and Morgan, 2004). Similar observations were found throughout the brain when sections were visualized at 0.1 µm intervals in the anteroposterior plane between the prefrontal cortex and cerebellum.

Anxiolytic Effects of Iron Oxide+ASV-30 Nanoparticles
Unconjugated APTES-coated Fe$_2$O$_3$ nanoparticle administration had no effect on anxiety-like behaviors or locomotion in the EPM (Figures 6A,B). There was no significant difference between vehicle- and nanoparticle-treated rats in time spent in the open arms of the EPM ($F_{(1, 20)} = 0.108, P = 0.746$) (Figure 6A). Furthermore, there was no significant difference in total distance moved between the treatment groups [$F_{(1, 20)} < 0.001, P = 0.996$; Figure 6B].

Increased anxiety-like behavior in amphetamine pretreated rats was reversed by ASV-30 delivered as nanoparticle cargo, without affecting locomotion in the EPM (Figures 6C,D). There was a significant interaction between pretreatment and treatment [$F_{(1, 48)} = 4.660, P = 0.036$] for time spent in open arms of the EPM. Rats pretreated with amphetamine and treated with iron oxide+vehicle spent less time in open arms than rats pretreated with saline and treated with iron oxide+vehicle ($P = 0.014$) indicating heightened anxiety-like behavior during amphetamine withdrawal (Figure 6C). However, amphetamine-pretreated rats receiving iron oxide+ASV-30 showed comparable time in open arms as compared to saline-pretreated rats receiving iron oxide+ASV-30 ($P = 0.614$) and exhibited greater time in open arms compared to amphetamine-pretreated rats receiving iron oxide+vehicle ($P = 0.001$; Figure 6C). Nanoparticle-ASV-30 treatment did not affect time spent in open arms for saline-pretreated animals ($P = 0.692$; Figure 6C). There was no significant effect of pretreatment [$F_{(1, 48)} = 0.831, P = 0.367$] or treatment [$F_{(1, 48)} = 0.009, P = 0.923$], and no significant interaction was found between pretreatment and treatment [$F_{(1, 48)} = 0.954, P = 0.334$] for distance moved in the EPM (Figure 6D).

DISCUSSION
APTES-coated Fe$_2$O$_3$ nanoparticles conjugated to ASV-30 were stable for at least 3 h in physiological conditions in vitro, and were observed throughout the brain 30 min following systemic administration in vivo. Thus, iron oxide nanoparticles were able to effectively cross the BBB without interference from conjugated ASV-30 in a time course where the peptide was still attached to the nanoparticle. The observation that iron oxide nanoparticles with ASV-30 were present in the brain within 30 min of systemic administration is similar timing observed for other nanoparticle delivery systems (Reimold et al., 2008; Ulbrich et al., 2009; Zensi et al., 2009).

Furthermore, iron oxide nanoparticles with ASV-30 were associated with neurons expressing CRF$_2$ receptors in the dRN
Iron oxide nanoparticles do not dissociate into ionic iron (Fe$^{3+}$) at physiological pH. (A) UV-vis spectrum of $1 \times 10^{-4}$ M Fe$^{3+}$ ions in solution shows two strong peaks at 275 and 209 nm. (B) UV-vis spectrum of iron oxide nanoparticles conjugated to FITC in aCSF solution do not show characteristic peaks at 275 and 209 nm, thus indicating that nanoparticles do not dissociate into oxygen and ionic iron (Fe$^{3+}$) in vivo within either 24 or 48 h.

Validation of ASV-30 conjugation with iron oxide surface. (A) FT-IR spectra of ASV-30 complexation with APTES-Fe$_2$O$_3$ coated nanoparticles as a function of time. (B) Comparison of FT-IR spectra of ASV-30-APTES-coated Fe$_2$O$_3$ nanoparticles washed with aCSF to unwashed ASV-30-APTES-Fe$_2$O$_3$ nanoparticle.
and lateral septum, both brain regions in which CRF₂ receptors have been associated with increased anxiety (Henry et al., 2006; Vuong et al., 2010; Bledsoe et al., 2011). There was little co-localization between the Fe₂O₃+ASV-30 complex and the CRF₂ receptor labeling. It is possible that this was a result of the Fe₂O₃+ASV-30 complex being taken up into CRF₂-containing cells. We are unaware of a transport mechanism that would allow these complexes to be up-taken into neurons, since any iron-related transport mechanism would require binding of ferric (Fe³⁺) iron (Moos et al., 2007) and thus the complex would have dissociated. Another possibility is that the majority of CRF₂ receptor antibody binding is intracellular and thus the binding site of the CRF₂ antibody is at different cellular location when compared to the Fe₂O₃+ASV-30 complex that presumably bound to the extracellular domain of the CRF₂ receptor. Iron oxide+ASV-30 nanoparticles were also localized near or with neurons that did not express CRF₂-expressing neurons, but did appear to be preferentially localized to cells rather than significant accumulation in extracellular space. This is consistent with the distribution noted for poly(n-butylcyano-acrylate) nanoparticles for example, which were observed to be distributed throughout the brain tissue and localized to neuronal and non-neuronal cells but little distribution in the extracellular space (Reimold et al., 2008). Future work will need to determine non-neural distribution and effects of the Fe₂O₃+ASV-30 complex in organs such as the kidney and liver, and in peripheral tissues that highly express CRF₂ receptor such as the heart, gastrointestinal...
FIGURE 6 | Iron oxide ASV-30 delivered as nanoparticle cargo is anxiolytic but does not affect locomotion. (A) Time spent in open arms of the elevated plus maze (EPM) did not differ between rats injected with vehicle and with APTES-coated iron oxide nanoparticles (87.7 µg/kg Fe₂O₃; ip) 30 min prior to testing. (B) APTES-coated iron oxide nanoparticle injections (87.7 µg/kg Fe₂O₃; ip) did not affect total locomotion in the EPM compared to vehicle controls. (C) Amphetamine pretreated rats (Amp) spend less time in the open arms of the elevated plus maze compared to saline pretreated rats (Sal) when both groups receive APTES-coated iron oxide nanoparticle+vehicle (87.7 µg/kg Fe₂O₃; ip) treatment 30 min prior to testing. The effects of amphetamine were reversed when APTES-coated iron oxide nanoparticle+ASV-30 (87.7 µg/kg Fe₂O₃ with 200 µg/kg ASV-30, ip) was administered 30 min prior to testing. (D) Neither amphetamine pretreatment or iron oxide nanoparticle administration affected locomotion in the EPM. # Significant difference (p < 0.05) compared to saline pretreated nanoparticle+vehicle group. * Significant difference (p < 0.05) compared to amphetamine pretreated nanoparticle+vehicle group.
receptors in the brain has no effect on the anxiety-like behavior of control rats (e.g., saline pre-treated or non-manipulated rats; Takahashi, 2001; Vuong et al., 2010; Bledsoe et al., 2011; Reinbold et al., 2014). However, an exception to this was the finding that a CRF2 receptor antagonist infused into the ventricles increased anxiety-like behavior of saline-pretreated rats but this effect was not apparent in rats that did not receive any pretreatment (Reinbold et al., 2014). Reinbold et al. show that the saline pre-treatment in that particular study resulted in alterations to the ratio of CRF1 to CRF2 receptors in the lateral septum that may have resulted in CRF2 receptor antagonism being anxiogenic in those rats. Together, current findings suggest a specific effect of systemically administered, nanoparticle-conjugated ASV-30 on heightened anxiety-like behavior in rats that mimics findings from intracranial infusions in the majority of studies. Future work should assess the concentration of ASV-30 that reaches the brain via this method of administration, and also test the utility of APTES-coated Fe3O4 nanoparticles for the delivery of other peptides to the brain. Furthermore, testing the ability of systemically-administered Fe3O4+ASV-30 to produce anxiolytic effects in different animal models, and with other tests of anxiety-like behaviors, and determining whether these effects are long-lasting are all necessary for understanding the generalizability of this complex as a potential anxiolytic.

A second major consideration for the future is determining any long-term cellular effects of the APTES-coated Fe3O4 nanoparticles described here. Small diameter uncoated Fe3O4 nanoparticles promote biofilm formation and bacterial growth in vitro (Borcherding et al., 2014), but is not known whether coated iron oxide nanoparticles as used here would have a similar effect in vivo. Dimercaptosuccinic acid (DSMA)-coated Fe3O4 nanoparticles reduce the ability of PC12 cells to respond to nerve growth factor and are pro-apoptotic in rat sciatic nerve (Pisanic et al., 2007; Kim et al., 2013). These previously observed neurotoxic effects are thought to be a result of the DSMA coating rather than effects of Fe3O4 alone. The treatment of nanoparticles with DSMA prevents nanoparticle aggregation and thus is used to improve cellular uptake, but the resultant increase in iron accumulation appear to increase generation of reactive oxygen species (ROS) by Fe3+(Moos and Morgan, 2004; Pisanic et al., 2009; Shubayev et al., 2009; Kim et al., 2013). The stability of the DSMA-free Fe3O4 nanoparticles in physiological pH described here suggests that formation of ROS in the brain is unlikely in response to nanoparticle administration in the current study. Furthermore, aminosilane coating (such as APTES) of Fe3O4 iron oxide nanoparticles does not affect cortical cell viability, which stands in contrast to other coatings such as poly-dimethylamine-co-epichlorhydrin-co-ethylendiamine (PEA) that induced neurotoxicity (Rivet et al., 2012). While APTES-coated Fe3O4 nanoparticles utilized here appear to have little reported toxicity, the in vivo cellular effects of these nanoparticles, including their ability to induce ROS, remains to be tested.

In conclusion, the current findings show that APTES-coated Fe3O4 nanoparticles represent a feasible delivery mechanism to transport a CRF-related peptide to the brain. Future work should focus on the generalizability of these findings to other peptides and should also determine the safety of chronic use of this nanomaterial. Further validation of the Fe3O4 nanoparticles described here as a stable system for delivery of peptide-based drugs to the brain has important implications for the study and treatment of a variety of neurological and psychiatric conditions where peptidergic systems are implicated.

AUTHOR CONTRIBUTIONS

GF, RK, and NV designed the research; NV, JS, CW, TK, and GF wrote the manuscript and performed the research; CW, TK, and RK contributed reagents; CW, RK, NV, and GF analyzed data; and all authors contributed to the editing of the manuscript.

FUNDING

This work was supported by NIH R01 DA019921, and University of South Dakota Nelson Faculty Research Grant (RK and GF). NV was supported by an U. Discover undergraduate research fellowship from the University of South Dakota.

ACKNOWLEDGMENTS

We thank Matthew Weber, Brenna Bray, Vyshnavi Kumaraswamy and Rajeshwari Solanki for their assistance in carrying out experimental procedures. We also thank Scott Oullette, Ph.D and Scott Patterson Ph.D, and the USD Imaging Core for their assistance with confocal microscopy; and Michael Watt, Ph.D and the USD Behavioral Core for assistance with behavioral testing.

REFERENCES

Bale, T. L. (2005). Sensitivity to stress: dysregulation of CRF pathways and disease development. *Horm. Behav.* 48, 1–10. doi: 10.1016/j.yhbeh.2005.01.009

Bale, T. L., and Vale, W. W. (2004). CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol.* 44, 525–527. doi: 10.1146/annurev.pharmaco.44.101802.121410

Barr, J. L., Renner, K., and Forster, G. L. (2010). Withdrawal from chronic amphetamine produces persistent anxiety-like behavior but temporally-limited reductions in monoamines and neurogenesis in the adult rat dentate gyrus. *Neuropsychopharmacology* 59, 395–405. doi: 10.1016/j.neuropsychopharm.2010.05.011

Basso, A. M., Spina, M., Rivier, J., Vale, W., and Koob, G. F. (1999). Corticotropin-releasing factor antagonist attenuates the anxiogenic-like effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats. *Psychopharmacology* 145, 21–30. doi: 10.1007/s002130051028

BatraKova, E. V., Vinogradov, S. V., Robinson, S. M., Niehoff, M. L., Banks, W. A., and Kabanov, A. V. (2005). Polypeptide point modifications with fatty acid and amphiphilic block copolymers for enhanced brain delivery. *Bioconjug. Chem.* 16, 793–802. doi: 10.1021/bc049730c

Bledsoe, A. C., Oliver, K. M., Scholl, J. L., and Forster, G. L. (2011). Anxiety states induced by post-weaning social isolation are mediated by CRF receptors in the dorsal raphe nucleus. *Brain Res. Bull.* 85, 117–122. doi: 10.1016/j.brainresbull.2011.03.003

Vinzant et al. - Peptide Delivery to the Brain

Frontiers in Neuroscience | www.frontiersin.org 8 November 2017 | Volume 11 | Article 608
Forster, G. L., Novick, A. M., Scholl, J. L., and Watt, M. J. (2012).

Higelin, J., Py-Lang, G., Paternoster, C., Ellis, G. J., Patel, A., and Dautzenberg, F.

Borcherding, J., Baltrusaitis, J., Chen, H., Stebounova, L., Wu, C.-M., Rubasinghe, G., et al. (2014). Iron oxide nanoparticles induce Pseudomonas aeruginosa growth, induce biofilm formation, and inhibit antimicrobial peptide function. Environ. Sci. Nano 1, 123–132. doi: 10.3389/fenvsci.2014.00029

Davies, J., Chen, J., Pink, R., Carter, D., Saunders, N., Sotiriadis, G., et al. (2015). Orexin receptors exert a neuroprotective effect in Alzheimer’s disease (AD) via heterodimerization with GPR103. Sci. Rep. 5:12584. doi: 10.1038/srep12584

Egleton, R. D., Mitchell, S. A., Huber, J. D., Janders, J., Stropova, D., Pott, R., et al. (2000). Improved bioavailability to the brain of glycosylated Met-enkephalin analogs. Brain Res. 881, 37–46. doi: 10.1016/S0006-8993(00)02794-3

Forster, G. L., Feng, N., Watt, M. J., Korzan, W. J., Mouw, N. J., Summers, C. H., et al. (2006). The role of the amygdala in anxiety disorders,” in The Amygdala - A Discrete Multitasking Manager, ed B. Ferry (Rejeka: InTech), 61–102.

Forster, G. L., Pringle, R. B., Mouw, N. J., Vuong, S. M., Watt, M. J., Burke, A. R., et al. (2008). Corticotropin-releasing factor in the dorsal raphe nucleus increases medial prefrontal cortical serotonin via type 2 receptors and median raphe nucleus activity. Eur. J. Neurosci. 28, 299–310. doi: 10.1111/j.1460-9588.2008.06333.x

Hare, D., Ayton, S., Bush, A., and Lei, P. (2013). A delicate balance: iron metabolism and diseases of the brain. Front. Aging Neurosci. 5:34. doi: 10.3389/fnagi.2013.00034

Henry, B., Vale, W., and Markou, A. (2006). The effect of lateral septum corticotropin releasing factor receptor 2 activation on anxiety is modulated by stress. J. Neurosci. 26, 9142–9152. doi: 10.1523/JNEUROSCI.1944-06.2006

Higelin, J., Py-Lang, G., Paternoster, C., Ellis, G. J., Patel, A., and Dautzenberg, F. M. (2001). 125I-Antisauvagine-30: a novel and specific high-affinity radioligand for the characterization of corticotropin-releasing factor type 2 receptors. Neuropharmacology 40, 114–122. doi: 10.1016/S0028-3908(00)00156-2

Holmes, A., Heilig, M., Rupniak, N. M., Steckler, T., and Griebel, G. (2003). Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. Trends Pharmacol. Sci. 24, 580–588. doi: 10.1016/j.tips.2003.09.011

Irwin, W. J., Dwivedi, A. K., Holbrook, P. A., and Dey, M. J. (1994). The effect of cyclocydrolins on the stability of peptides in nasal enzymic systems. Pharm. Res. 11, 1698–1703. doi: 10.1023/A:101894682225

Jain, K. K. (2012). Nanobiotechnology-based strategies for crossing the blood-brain barrier. Nanomedicine 7, 1225–1233. doi: 10.2217/nmn.12.86

Karatas, H., Aktas, Y., Gursoy-Ozdemir, Y., Bodur, E., Yemisci, M., Cahan, S., et al. (2009). A nanomedicine transports a peptide caspase-3 inhibitor across the blood-brain barrier and provides neuroprotection. J. Neurosci. 29, 13761–13769. doi: 10.1523/JNEUROSCI.4246-09.2009

Kikusui, T., Takeuchi, Y., and Mori, Y. (2000). Involvement of K-ligands in the release of corticotropin releasing factor from the amygdala. J. Neurosci. 20, 1376–1383. doi: 10.1523/JNEUROSCI.2506-00.2000

Kikutani, H., Kikusui, T., Takeuchi, Y., and Mori, Y. (2000). Improved bioavailability to the brain of glycosylated Met-enkephalin analogs. Brain Res. 881, 37–46. doi: 10.1016/S0006-8993(00)02794-3

Lalatsa, A., Schatzlein, A. G., and Uchegbu, I. F. (2014). Strategies to deliver peptide drugs to the brain barrier and drug delivery to the CNS. BMC Neurosci. 9:54. doi: 10.1186/1471-2202-9-S4

Lukkes, J., Vuong, S., Scholl, J., Oliver, H., and Forster, G. (2009). Corticotropin-releasing factor receptor antagonism within the dorsal raphe nucleus reduces social anxiety-like behavior after early-life social isolation. J. Neurosci. 29, 9955–9960. doi: 10.1523/JNEUROSCI.0854-09.2009

Meyer, D. L., Davies, D. R., Barr, I. L., Manzerra, P., and Forster, G. L. (2012). Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. Exp. Neurol. 235, 574–587. doi: 10.1016/j.expneurol.2012.03.012

Minchin, R. F., and Martin, D. J. (2010). Minireview: nanoparticles for molecular imaging – an overview. Endocrinoology 151, 474–481. doi: 10.1210/en.2009-1012

Moos, T., and Morgan, E. H. (2004). Growth and electrophysiological properties of rat embryonic cardiomyocytes on hydroxy- and carboxyl-modified surfaces. J. Biomater. Sci. Polym. 19, 1319–1331. doi: 10.1163/156862087X52399

Nemeroff, C. B. (2004). Neurobiological consequences of childhood trauma. J. Clin. Psychiatry 65(Suppl. 1), 18–28.

Park, H. J., Ran, Y., Jung, J. L., Holmes, O., Price, A. R., Smithson, L., et al. (2015). The stress response neuropeptide CRF increases amyloid-beta production by regulating gamma-secretase activity. EMBO J. 34, 1674–1686. doi: 10.15252/embj.201488795

Pernar, L., Curtis, A. L., Vale, W. W., Rivier, J. E., and Valentino, R. J. (2004). Selective activation of corticotropin-releasing factor-2 receptors on neurochemically identified neurons in the rat dorsal raphe nucleus reveals dual actions. J. Neurosci. 24, 1305–1311. doi: 10.1523/JNEUROSCI.2885-03.2004

Plakkala-Kanthikeel, S., Atuiri, V. S., Sagar, V., Saxena, S. K., and Nair, M. (2013). Targeted brain derived neurotrophic factors (BDNF) delivery across the blood-brain barrier for neuro-protection using magnetic nano carriers: an in-vitro study. PLoS ONE 8:e62241. doi: 10.1371/journal.pone.0062241

Rivet, C. J., Yuan, Y., Borca-Tasciuc, D. A., and Gilbert, R. J. (2012). Altering iron oxide nanoparticle surface properties induce cortical neuron cytotoxicity. Chem. Res. Toxicol. 25, 153–161. doi: 10.1021/tr200369s

Rivest, I., Domke, D., Bender, J., Seyfried, C. A., Radunz, H. E., and Fricker, G. (2008). Delivery of nanoparticles to the brain detected by fluorescence microscopy. Eur. J. Pharm. Biopharm. 70, 627–632. doi: 10.1016/j.ejpb.2008.05.007

Silva, G. S. (2008). Nanotechnology approaches to crossing the blood-brain barrier and drug delivery to the CNS. BMJ. Neurosci. 93:45. doi: 10.1186/1471-2202-9-S3-54

Spencer, B., Potkar, R., Metcalf, J., Thrin, I., Adame, A., Rocken, E., et al. (2016). Systemic central nervous system (CNS)-targeted delivery of neuropeptide Y (NPY) reduces neurodegeneration and increases neural precursor cell proliferation in a mouse model of Alzheimer disease. J. Biol. Chem. 291, 1905–1920. doi: 10.1074/jbc.M115.671815

Takahashi, L. K. (2001). Role of CRF(1) and CRF(2) receptors in fear and anxiety. Neurosci. Biobehav. Rev. 25, 627–636. doi: 10.1016/S0149-7634(01)00046-X
Teixido, M., Belda, E., Zurita, X., Llora, M., and Fabre, S. (2005). Evolutionary combinatorial chemistry, a novel tool for SAR studies on peptide transport across the blood-brain barrier. Part 2. design, synthesis and evaluation of a first generation of peptides. J. Pept. Sci. 11, 789–804. doi: 10.1002/psc.679

Tu, W., Cook, A., Scholl, J. L., Mears, M., Watt, M. I., Renner, K. J., et al. (2014). Serotonin in the ventral hippocampus modulates anxiety-like behavior during amphetamine withdrawal. Neuroscience 281, 35–43. doi: 10.1016/j.neuroscience.2014.09.019

Ulbrich, K., Hekmatara, T., Herbert, E., and Kreuter, J. (2009). Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood-brain barrier (BBB). Eur. J. Pharm. Biopharm. 71, 251–256. doi: 10.1016/j.ejpb.2008.08.021

Vuong, S. M., Oliver, H. A., Scholl, J. L., Oliver, K. M., and Forster, G. L. (2010). Increased anxiety-like behavior of rats during amphetamine withdrawal is reversed by CRF2 receptor antagonism. Behav. Brain Res. 208, 278–281. doi: 10.1016/j.bbr.2009.11.036

Yang, H. (2010). Nanoparticle-mediated brain-specific drug delivery, imaging and diagnosis. Pharm. Res. 27, 1759–1771. doi: 10.1007/s11095-010-0141-7

Zensi, A., Begley, D., Pontikis, C., Legros, C., Mihoreanu, L., Wagner, S., et al. (2009). Albumin nanoparticles targeted with Apo E enter the CNS by transcytosis and are delivered to neurons. J. Control. Release 137, 78–86. doi: 10.1016/j.jconrel.2009.03.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer TS and handling Editor declared their shared affiliation.

Copyright © 2017 Vinzant, Scholl, Wu, Kindle, Koodali and Forster. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.