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Brain Changes Associated with Thromboxane Receptor Antagonist, SQ 29,548, Treatment in a Mouse Model

Andrew A. Rebel*, Siri A. Urquhart*, Kendra L. Puig, Atreyi Ghatak, Stephen A. Brose, Mikhail Y. Golovko, and Colin K. Combs

*Department of Biology and Department of Basic Sciences, University of North Dakota, School of Medicine & Health Sciences, Grand Forks, North Dakota 58203

Abstract

The purpose of this study was to characterize behavioral and physiological effects of a selective thromboxane receptor (TP) antagonist, SQ 29,548, in the C57BL/6 mouse model. At six months of age, male mice were given either sham or drug intraperitoneal injections for three days at a dose of 2mg/kg each day. On the day after the final injection mice were subjected to behavioral testing paradigms before brain collection. Left hemisphere hippocampi were collected from all mice for protein analysis via western blot. Right brain hemispheres were fixed and imbedded in gelatin, and serially sectioned. The sections were immunostained using anti-c-Fos antibodies. Prostaglandin analysis was performed from remaining homogenized brain samples, minus the hippocampi. Injection of SQ 29,548 decreased selective brain prostaglandin levels compared to sham controls. This correlated with robust increases in limbic region c-Fos immunoreactivity in the SQ 29,548 injected mice. However, drug treated mice demonstrated no significant changes in relevant hippocampal protein levels compared to sham treatments, as determined by western blots. Surprisingly, injection of SQ 29,548 caused mixed changes in parameters of depression and anxiety-like behavior in the mice. In conclusion, the results indicate that administration of peripheral TP receptor antagonists alters brain levels of prostanoids and influences neuronal activity with only minimal alterations of behavior. Whether the drug affects neurons directly or through a secondary pathway involving endothelium or other tissues remains unclear.

Keywords

thromboxane; depression; anxiety; c-Fos; thromboxane receptor

Introduction

It is well known that thromboxane A₂ (TXA₂) is a potent stimulus of platelet activation which causes changes in platelet shape, aggregation, and secretion (Hamberg et al. 1975). TXA₂ and other prostanoids (PG) are formed when arachidonic acid is released from the phospholipid bilayer and metabolized by cyclooxygenases (Simmons et al. 2004). In

Correspondence to: Colin K. Combs, PhD, Department of Basic Sciences, University of North Dakota School of Medicine & Health Sciences, Grand Forks, North Dakota 58203. colin.combs@med.und.edu.

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general, PG that are produced from this reaction are either pro-inflammatory or anti-inflammatory in nature, and elicit their effects in a wide variety of tissues. These effects are mediated through a multitude of prostanoid receptors of varying binding specificity (Eglen and Whiting 1988). These receptors are of particular interest, as they might be therapeutically targeted to treat a wide variety of inflammation-related conditions.

Although it remains mechanistically unclear as well as somewhat controversial, numerous studies continue to report a positive correlation between increased platelet activation and depression in humans (Aschbacher et al. 2009; Berk and Plein 2000; Canan et al. 2012; Gehi et al. 2010; Huang et al. 2014; Lederbogen et al. 2004; Lopez-Vilchez et al. 2014; Morel-Kopp et al. 2009; Moreno et al. 2013; Musselman et al. 1996; Neubauer et al. 2013; Williams 2012; Williams et al. 2014). The increased inflammatory state associated with platelet activation may well include changes in pro-aggregation PG production. For example, a 1983 clinical study by Lieb *et al.* showed that in thirty patients suffering from depression, all had markedly increased levels of plasma thromboxane (Lieb et al. 1983). Elevated levels of thromboxane inherently indicate associated, mild tissue inflammation. This type of stress-related inflammation has been studied extensively with regard to cytokines which are thought to be a driving stimulus behind depression pathophysiology (Kim et al. 2007; Zeugmann et al. 2010). However, a 2008 study noted that brain levels of PGE₂, another pro-inflammatory prostaglandin, were reduced in rats with mood disorders following successful drug treatment (Tassoni et al. 2008). These data suggest that in addition to cytokines, prostaglandins may have a role in regulating brain changes during depression. Based upon the numerous studies demonstrating a positive correlation between depression behavior and platelet activation as well as some limited evidence of elevated levels of plasma thromboxane in depressed patients, we determined whether an anti-platelet drug strategy such as a thromboxane receptor antagonist, could modulate depression-like behavior. This was particularly compelling given the fact that receptor antagonists have proven safe in human trial (Bousser et al. 2011).

Manipulation of the TXA₂ pathway is typically accomplished either through receptor antagonists, or inhibition of TXA₂ synthesis. Human studies have verified that both inhibition of TXA₂ synthesis and TXA₂ receptor antagonists are viable strategies for manipulating this pathway (Bousser et al. 2011; Reilly and FitzGerald 1987). Although receptor antagonism is therapeutically attractive, selective small molecule discovery has been problematic due to varying binding specificities and affinities for the receptor. However, the TXA₂ receptor antagonist, SQ 29,548, binds with high specificity to the thromboxane A₂/prostaglandin H₂ (TP)-receptor thus representing a useful preclinical reagent (Hedberg et al. 1988; Ting et al. 2012). Previous analyses of the drug have shown that, when compared to other thromboxane receptor antagonists, SQ 29,548 binds far more selectively as well as with higher affinity than numerous other TP receptor antagonists, including SQ 28,668, SQ 30,741, BM 13,177, and BM 13,505 (Hedberg *et al.*, 1988). Further, a 1985 study by Darius *et al.* demonstrated that thromboxane synthesis was not significantly impacted after treatment with SQ 29,548 (Darius et al. 1985). Based upon these favorable properties, we elected to use this agent to determine whether antagonizing the TP receptor would alter anxiety or depression-like behavior in mice. Using the well-

characterized forced swim, open field, elevated zero maze, and hanging tail suspension tests we quantified effects of SQ 29,548 on anxiety and depression-like behavior in male C57BL/6 mice.

Materials and Methods

Materials

Anti- β -amyloid precursor protein (APP) antibody was purchased from Invitrogen (Carlsbad, CA, USA). Anti-rabbit (goat), anti-goat (bovine), anti-rat (goat), and anti-mouse (bovine) horseradish peroxidase-conjugated secondary antibodies, and anti- β actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Thromboxane A₂ (TXA₂) Receptor, anti-COX-1, and anti-COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-TXA₂ synthase and anti-BDNF antibodies were purchased from Abcam Inc (Cambridge, MA, USA). Anti-iNOS antibody was purchased from Alexis Biochemicals (San Diego, CA, USA). Anti-IBA-1 antibody was purchased from Wako Chemicals (Osaka, Japan). Anti-GFAP and anti-PSD95 antibodies were purchased from Cell Signaling Technology Inc (Danvers, MA, USA). Anti-synaptophysin antibody was purchased from Chemicon International Inc (Temecula, CA, USA). Anti-c-Fos antibody was purchased from Novus Biologicals (Littleton, CO, USA). Antibody binding in brain was visualized using Vector VIP as chromogens (Vector Laboratories, Burlingame, CA, USA). PG deuterated standards were purchased from Cayman Chemicals (Ann Arbor, MI).

Animal Groups

At six months of age 13 male C57BL6 mice were treated for 3 days with intraperitoneal injection of DMSO vehicle each day and 13 male C57BL6 mice were treated for 3 days with the TP receptor antagonist, SQ 29,548, at 2 mg/kg each day. On the 4th day the animals were given 4 behavioral tests (open field testing, tail suspension test, elevated zero maze, and forced swim test) before brain collection. Animal use was performed in accordance with protocols approved by the University of North Dakota IACUC.

Open Field Test

The open field test was performed in order to assess any general locomotion or anxiety changes associated with TP receptor antagonist treatment. A 19cm $\times$ 45cm $\times$ 25cm container was used to allow the mouse to freely move and explore for 6 minutes. Quantification of immobile versus mobile time was performed from video capture to assess general locomotive activity via Anymaze software (Stoelting Co. Wood Dale, IL, USA). Following the test, the animals were placed back into their home cages.

Tail Suspension Test

The tail suspension behavioral test was performed in order to assess the effect of TP receptor antagonist treatment on depression behavior. Adhesive tape was placed approximately 2cm from the end of the tail and then wrapped around the horizontal bar placed 50cm above the bench, ensuring that the tail was straight and the abdomen faced the camera. The mouse was in tail suspension for 6 minutes. Animals were considered immobile when they hung

passively and motionless. Immobility is regarded as an indication of depressed behavior in this paradigm. Periods of immobility versus mobility were quantified from video capture of animal tests via Anymaze software (Stoelting Co. Wood Dale, IL, USA). Following tail suspension, the tape was removed and the animal placed back in its home cage.

Forced Swim Test

The forced swim behavioral test was performed in order to assess the effect of TP receptor antagonist treatment on depression behavior. A 20cm high by 15cm diameter container with water (temperature 23-38°C) at a level of 12cm was used. Mice were gently placed in the water for 6 minutes with the first 2 minutes being used as the acquisition time and the remaining 4 minutes for scoring behavioral activity. Periods of immobility or floating were equated with depressed behavior. Immobile and mobile times were quantified from video capture via Anymaze software (Stoelting Co. Wood Dale, IL, USA). Following removal from the swimming chamber, the animals were dried off with a towel and placed in a cage under a heat lamp to allow the animals to dry completely before being transferred back to their home cages.

Elevated Zero Maze

Elevated zero maze was used to measure anxiety behavior. The zero maze (Stoelting Co., IL) was made up of grey plastic/plexiglass material, 48 inches in height, 3.5 inches wide, and with an inner diameter of 20 inches. The maze had 2 compartments enclosed by two 7 inches tall walls made of the same color material (closed arms of the maze). The 2 closed arms were equidistant to each other, and alternated by open areas without walls (open arms of the maze). The maze was set on the floor of the testing room, and the height of the maze served as an aversive stimulus for the animals. Each mouse was placed in either of the closed arms (with alternating the sides) and was observed and recorded with an overhead digital video camera for 10 minutes. The videos were later re-observed, scored, and analyzed by measuring time spent by the animals in open and closed arms, and number of entries to each.

Western Blotting

At the conclusion of behavioral testing, all mice in the tested groups were euthanized and perfused with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS and the left hemisphere hippocampi were collected for protein analysis. The tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer, quantitated by Bradford assay (Bradford 1976), and lysates were resolved by 10% SDS-PAGE and transferred to polyvinyl difluoride membranes (Pall Corporation, Pensacola, FL) for western blotting. The membranes were blocked and then incubated with primary antibodies overnight at 4°C. Blots were washed, incubated with horseradish peroxidase conjugated secondary antibodies, washed and binding detected via enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA). Western blots were quantified using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA). Optical density of bands were normalized against their respective loading controls and averaged ($\pm$ SD).

Immunohistochemistry

Right brain hemispheres from animals were immersion fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brain hemispheres were embedded in gelatin and serially sectioned (40µm) via freezing microtome. Sections were immunostained using anti-c-Fos antibodies. Antibody binding was visualized using Vector VIP (Vector Laboratories, Burlingame, CA, USA) as the chromogen. The sections were mounted onto double subbed glass slides, dehydrated through a series of ethanol solutions and Histo-Clear (National Diagnostics, Atlanta, GA, USA), then glass coverslipped using VectaMount (Vector Laboratories, Burlingame, CA, USA). Images were taken using an upright Leica DM1000 microscope and Leica DF320 digital camera system (Leica Microsystems, Wetzlar, Germany). Figures were made using Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA, USA). Numbers of c-Fos positive cells were counted from the entire visible nucleus (medial orbital cortex, piriform cortex, posterior hypothalamic cortex, posterior cortical amygdala) from all sections containing the nuclei from n=8-10 mice per condition.

Prostanoid Analysis

PG were analyzed as previously described (Golovko and Murphy 2008). Remaining brain samples from the left hemisphere minus the hippocampi were homogenized in 1 mL of saline and extracted with 2 mL of acetone containing 0.005% butylated hydroxytoluene (BHT) followed by 2 mL of chloroform with 0.005% BHT. PGE₂d₄ was used as internal standard. After extraction, the samples were dried under a stream of nitrogen and then redissolved in 30 µL of acetonitrile:water (1:2 by volume). Reverse-phase LC electrospray ionization mass spectrometry was used for PG analysis. The PG were separated on a Luna C-18(2) (3 µm column, 100 Å pore diameter, 150 × 2.0 mm) (Phenomenex, Torrance, CA, USA) with security guard cartridge system (Phenomenex, Torrance, CA, USA). The LC system consisted of an Agilent 1100 series LC pump with a wellplate autosampler (Agilent Technologies, Santa Clara, CA, USA). The solvent system was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate was 0.2 mL/min. The separation program started with 10% of solvent B. At 2 min, the percentage of B was increased to 65% over 8 min, at 15 min the percentage of B was increased to 90% over 5 min, and at 35 min it was reduced to 10% over 2 min. Equilibration time between runs was 13 min. Mass spectrometry analysis was performed using a quadrupole mass spectrometer (API3000, Applied Biosystem, Foster City, CA, USA) equipped with a TurboIonSpray ionization source. Analyst software version 1.5.1 (Applied Biosystem, Foster City, CA, USA) was used for instrument control, data acquisition, and data analysis. The mass spectrometer was optimized in the multiple reaction-monitoring mode. The source was operated in negative ion electrospray mode at 450 °C, electrospray voltage was -4250 V, nebulizer gas was 8 L/min and curtain gas was 11 L/min. Declustering potential, focusing potential, and entrance potential were optimized individually for each analyte. The quadrupole mass spectrometer was operated at unit resolution.

Statistical Analysis

Statistical significance was calculated by an unpaired two-tailed t-test with or without a Welch correction as needed. When the data failed the Kolmogorov-Smirnov normality test

(c-Fos counts) it was analyzed by a Mann-Whitney Rank Sum test. Pearson correlation was used to measure the strength of the linear relationship between open field or hanging tail suspension behavioral changes and total brain PG levels in individual mice injected three days.

Results

IP injection of the TP receptor antagonist, SQ 29,548, decreased brain prostaglandin levels in mice

In order to determine whether peripheral modulation of TP receptor activity would alter brain levels of PG, we elected to use the well characterized TP receptor antagonist, SQ 29,548. SQ 29,548 has a reported 94 to 97% binding specificity under equilibrium conditions at 5 nM concentration and an IC_{50} of 4.51 ± 0.26 nM (Hedberg et al. 1988). A dose of 2 mg/kg injected three days was used to study the effects of the antagonist without causing brain accumulation (Soper et al. 2012). Brain levels of the prostaglandins, PGD_2 and PGE_2 but not $PGF_{2\alpha}$, were significantly reduced by three days of drug treatment compared to sham injected animals (Fig. 1). Levels of TXB_2 , the stable metabolite of TXA_2 , were also significantly reduced in the drug treated mouse brains compared to sham injected controls (Fig. 1). However, similar to $PGF_{2\alpha}$, levels of $6ketoPGF_{1\alpha}$, the stable degradation product of prostacyclin PGI_2 , were not affected. Summed together, overall PG levels were significantly attenuated in the drug injected mouse brains compared to sham animals indicating that peripheral antagonism of the TP receptor significantly and selectively altered brain PG biology.

TP receptor antagonist injection had variable effects on anxiety and depression-like behavior in mice

Based upon the decreases in brain prostaglandins observed following TP receptor antagonist treatment of the mice it was reasonable to expect that these animals would exhibit consequential behavioral changes affecting depression or anxiety. In order to assess the effect of peripheral SQ 29,548 administration on behavior, multiple assessments of depression and anxiety-like behavior were performed including open field, hanging tail suspension, elevated zero maze, and forced swim tests (Belzung and Griebel 2001; Cook et al. 2001; Crawley and Paylor 1997; Hall 1936; Prut and Belzung 2003; Simon et al. 1994). The drug treated mice performing in the open field test had overall no robust change in locomotion or time in the center but had fewer clockwise and total body rotations (Fig. 2). Interestingly, SQ 29,548 injected mice showed a significant decrease in thigmotaxis evidenced by decreased entries into all four corner quadrants (Fig. 2) in correlation with decreased brain PG levels (Fig. 1). Although decreased thigmotaxis can be interpreted as reduced anxiety, the lack of difference in time spent in the center of the maze suggests no overall robust change in anxiety behavior following three days of SQ 29,548 injection. To further address a possible drug-dependent change in anxiety-related behavior, SQ 29,548 injected mice were examined via an additional anxiety assessment paradigm, the elevated zero maze. However, there was no difference between SQ 29,548 and sham injected mice with respect to time spent in open arms of the maze following SQ 29,548 injection (Fig. 3).

Based upon the fact that three days of SQ 29,548 injection did attenuate TXB₂, PGD₂, and PGE₂ levels in treated animals and subtle changes in thigmotaxis were observed, we next examined whether changes in depression-associated behavior resulted from three days of drug treatment. This was quantitated via the hanging tail suspension and forced swim tests (Porsolt et al. 1977a; Porsolt et al. 1977b; Steru et al. 1985). Surprisingly, drug treated mice evaluated in the hanging tail suspension test spent more time immobile and had fewer start/stop events supporting increased depression-like behavior (Fig. 4). However, a decrease in mobile time was not observed when the mice underwent the forced swim test (Fig. 5). In fact, the only change observed from SQ 29,548 injected mice in the forced swim test was an increase in counterclockwise body rotation suggesting no increase in depression following drug treatment as assessed by forced swim (Fig. 5).

As a final means of assessing behavioral changes following SQ 29,548 injection, the Pearson's correlation coefficient was calculated comparing individual total brain PG levels with individual open field and hanging tail suspension performances from the 3 day injected mice. Several significant changes were found for the SQ 29,548 but not the sham injected mice indicating, again, subtle effects of drug treatment on behavior (Fig. 6). Specifically, there was a negative linear relationship between total prostaglandins and Q4 entries, as the total amount of prostaglandins increased, the number of Q4 entries decreased in SQ 29,548 injected mice. There were no significant correlations with other quadrant entries. There was also a negative linear relationship between total prostaglandins and open field max speed, as the total amount of prostaglandins increased, the maximum speed at which the animal traveled in the open field decreased. Finally, there was a positive linear relationship between total prostaglandins and tail suspension immobile episodes, as the total amount of prostaglandins increased, the number of immobile episodes occurring during the tail suspension test increased (Fig. 6). Collectively, in spite of the many significant correlations following SQ 29,548 injection, overall behavioral testing demonstrated no uniform change in either anxiety or depression-like behavior following drug treatments in spite of several individual parameter correlations between specific behavioral tests and brain PG levels.

Western blots demonstrated no significant changes in relevant hippocampal protein levels in TXAR injected mice

Although the concentration of SQ 29,548 used was well below the level that allows significant brain accumulation (Soper et al. 2012), it was clearly sufficient to have brain effects on PG levels as well as behavioral outcomes. This was presumably due to inhibition of peripheral TP receptors. Based upon this ability of apparent peripheral TP receptor antagonism to affect the brain and the critical role of the hippocampus in limbic function, we next examined protein levels of synaptic and glial markers in the SQ 29,548 and sham injected mice. There were no significant changes in levels of presynaptic synaptophysin or postsynaptic PSD95 levels in either drug or sham injected mice (data not shown). Protein levels of reactive glial markers, GFAP and IBA-1, to examine astrocytes and microglia respectively, were also not altered by drug injection (data not shown). Changes in the proinflammatory marker, iNOS, or PGH₂ synthesis enzymes, COX-1, COX-2, and downstream thromboxane synthase were also not significantly different between drug and sham injected mice (data not shown). Similarly, no changes in TP receptor protein levels

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were observed in drug injected mice compared to sham controls (data not shown). As a further assessment of attenuated depression-associated behavior, hippocampal BDNF levels were examined based upon the well characterized role of decreased BDNF levels correlating with increased depression (Castren and Rantamaki 2010; Castren et al. 2007; Duman et al. 1997; Krishnan and Nestler 2008; Nibuya et al. 1995). However, no differences in BDNF levels were observed between SQ 29,548 and sham injected mice (data not shown). Finally, based upon recent evidence that TP receptor stimulation attenuates APP levels, we quantified any changes in drug or sham injected mice. As expected, SQ 29,548 injection had no effect on hippocampal APP levels consistent with a lack of effect on any of the proteins examined (data not shown). Therefore, there were no changes in any hippocampal protein markers examined to correlate with the drug-induced decrease in brain PG levels and slight alterations of performance in anxiety/depression testing.

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Brains from TP receptor antagonist injected mice demonstrated robust increases in limbic region c-Fos immunoreactivity

In order to understand whether the drug-dependent changes in brain prostanoid levels altered activity of various brain regions, neuronal immunoreactivity of the immediate early gene, c-Fos, was examined as an indirect assessment of neuron activity. Fixed brains sections from different brain regions from the mice injected three days with SQ 29,548 were immunostained using anti-c-Fos antibody. As expected, brains of SQ 29,548 injected mice demonstrated robust increases in c-Fos immunoreactivity in discrete limbic structures compared to sham injected mice (Fig. 7). Increased immunoreactivity was observed in the medial orbital cortex, piriform cortex, posterior hypothalamic area, and posterior cortical amygdala of SQ 29,548 injected mouse brains compared to sham animals (Fig. 7).

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Discussion

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TXA₂ has been shown to be a potent stimulus of platelet activation and tissue inflammation (Hamberg et al. 1975). Multiple TP receptor subtypes have been reported and are appealing targets for therapeutic intervention of inflammation-related pathology. Based upon the extensive literature demonstrating a proinflammatory, hypercoagulable platelet activation state positively correlating with depression behavior in humans (Aschbacher et al. 2009; Berk and Plein 2000; Canan et al. 2012; Gehi et al. 2010; Huang et al. 2014; Lederbogen et al. 2004; Lopez-Vilchez et al. 2014; Morel-Kopp et al. 2009; Moreno et al. 2013; Musselman et al. 1996; Neubauer et al. 2013; Williams 2012; Williams et al. 2014) and the modest literature suggesting elevated pro-thrombotic TXA₂ levels in depressed patients (Lieb et al. 1983), we examined whether a TP receptor antagonist would modulate depression and anxiety behavior in mice. We tested whether a highly selective TP receptor antagonist, SQ 29,548, would attenuate depression-like behavior in mice observed using the forced swim, and hanging tail suspension tests and attenuate anxiety in open field and elevated zero maze tests. Unexpectedly, peripheral drug delivery not only demonstrated a time-dependent decrease of brain PG levels but also had mixed effects on decreasing or increasing anxiety and depression behavior, respectively, depending upon the behavioral assessment. Perhaps not surprisingly, ip injected SQ 29,548 treatment correlated with specific activation of limbic regions of the brain detected by increased c-Fos

immunoreactivity. Therefore, in spite of the fact that drug injection altered brain PG levels and apparently also activated particular nuclei in the brain this did not appear to produce robust behavioral changes with respect to anxiety or depression. These data may support efforts to develop TXR antagonist therapies as well as provide a rationale for understanding regulatory mechanisms of brain PG production.

Several TP receptor agonists and antagonists have been investigated relative to their roles in the potential treatment of cardiovascular disease (Fu et al. 2008b; Singh et al. 1997; Viles-Gonzalez et al. 2005). These studies utilized different TP receptor antagonists and established that at minimum, all were effective in reducing inflammation during cardiovascular episodes. Our results on attenuation of PG levels upon treatment with TP receptor antagonist are consistent with these findings and provide a possible mechanism on attenuated inflammatory response. However, to date, no study was found that has sought to specifically compare TP receptor antagonism with behavior alterations in any model organism, though numerous studies note increased platelet activation associated with depression (Aschbacher et al. 2009; Berk and Plein 2000; Canan et al. 2012; Gehi et al. 2010; Huang et al. 2014; Lederbogen et al. 2004; Lopez-Vilchez et al. 2014; Morel-Kopp et al. 2009; Moreno et al. 2013; Musselman et al. 1996; Neubauer et al. 2013; Williams 2012; Williams et al. 2014). It is possible that the behavior noted in our study is associated with only acute SQ 29,548 treatment as opposed to chronic. Future study comparing both acute as well as chronic SQ 29,548 treatment, as would be required for treatment of cardiovascular disease, is necessary to better understand the potential behavioral side effects we observed. In addition, due to the fact that only two out of the four tests showed any behavioral alterations due to drug treatment and these two were somewhat conflicting with one another, it is likely that additional, perhaps more sensitive behavioral assessments would reveal whether anxiety or depression are significantly affected by peripheral TP receptor antagonist therapy. Indeed, the fact that no robust adverse side effects have been reported from clinical trials using TP receptor antagonists suggests that any changes in decreasing anxiety or increasing depression behavior are likely to be quite modest or nonexistent in humans (Bousser et al. 2009; Bousser et al. 2011; Davi et al. 2012; Fiessinger et al. 2010; Lesault et al. 2011).

Nevertheless, the fact that peripheral TP receptor antagonism resulted in a time-dependent decrease in brain PG levels remains a novel and exciting observation. One intriguing possibility is that the changes in the brain that we observed were due to SQ 29,548 effects on brain vasculature. The role of thromboxane and other arachidonic acid metabolites on vascular disease have been very well characterized as playing a significant role in chronic inflammation. Thromboxane has been shown to be elevated in cardiovascular disease states that promote endothelial dysfunction (Ellinsworth et al. 2014; Feng et al. 2011) and antagonism of the TP receptor is a promising target for treatment of those disease states (Feletou et al. 2011). Patient trials with the TP receptor antagonist, Terutroban, revealed that drug treatment chronically improves endothelium-dependent vasodilation and inhibits platelet aggregation, both of which are important in the prevention of cardiovascular episodes (Lesault et al. 2011). However, thromboxane plays a role in many other types of vasculature, and is not limited to the coronary conditions (Dong et al. 2013; Radenkovic et

al. 2010). Although this study did not assess changes in brain vasculature phenotype, future work will clearly require this.

The mechanistic causes of clinical depression have received much attention in recent years, due to the increased prevalence of depression in association with inflammation-related disease. Several studies have shown that reduced blood flow correlates with depression-like symptoms (Cooper et al. 2010; Cooper et al. 2011; d'Audiffret et al. 2010; Isingrini et al. 2011; Lavoie et al. 2010; Nelson et al. 1995; Neu et al. 2004; Peuler et al. 2012; Sugawara et al. 2010). The results obtained from our behavioral observations were that drug treatment increased depression-like behavior in the hanging tail suspension test but had no effect on depression in the forced swim test. Moreover, open field testing showed a decrease in anxiety while elevated zero maze testing demonstrated no effect of drug treatment on anxiety behavior. These contradictory findings are likely due to test sensitivity and variability in the usefulness of the animal tests for assessing human-relevant behavior. For example, Dedic et. al have reported that both hanging tail suspension and forced swim tests assess the response to an acute inescapable stress that provokes despair-based behavior and immobility instead of depression-like behavior (Dedic 2011). Depression in humans is characterized by prolonged feelings of helplessness and despair that likely cannot be fully replicated in acute testing with animal models. In addition, a 2000 study noted that the forced swim test can be used as an assessment measure for stress-induced anxiety, rather than depression (Gardier and Bourin 2001). Clearly additional testing with higher animal numbers, different treatment paradigms, and more sensitive tests of depression, in particular, may offer insight into whether peripheral TP receptor antagonism truly alters behavior.

It is well known that TP receptors are expressed in neurons, both in the central nervous system and in the periphery (Andoh et al. 2007; Carrithers et al. 1994; Fu et al. 2008a; Nakayama et al. 1997; Wacker et al. 2012). The known localization of TP receptors on neurons, coupled with the behavioral alterations noted in this study, suggest that the antagonist actions may indeed work through a neural pathway. Even though we observed behavioral and histological consequences following SQ 29,548 injection and neurons express TP receptors, it is still likely that these changes were independent from direct drug effects in the brain. A 2012 study by Soper *et al.* assessing effects of multiple TP antagonists revealed that a treatment dose of 5mg/kg via IP injection provided brain levels of SQ 29,548 that were below the limit of detection (Soper et al. 2012). This finding suggests that our ip injection of 2mg/kg per day was insufficient for penetration of the blood brain barrier (BBB) and the effects we observed are due to inhibition of peripheral TP receptors. Nevertheless, our injection paradigm for three days of delivery did result in attenuated brain PG levels, increased c-Fos staining, and mixed behavioral responses suggesting that either the drug accumulated sufficiently for brain penetration over that time or the treatments affected peripheral cells/tissues to produce the altered lipid metabolism, neuronal activity, and behavior.

Indeed, a variety of studies have demonstrated a range of physiologic consequences of TP receptor antagonists or agonists outside of the brain (Darius et al. 1985; Dong and Jones 1982; Eglén and Whiting 1988; Ogletree et al. 1985; Saraswathi et al. 2013). The multitude of physiological responses involving multiple cell types affected by TP receptor activation

or inhibition in peripheral tissue could be responsible for the ultimate changes in neuronal c-Fos expression and behavioral changes we observed. It is difficult to correlate the changes in c-Fos immunoreactivity observed with either the decreases in brain PG or the behavior changes. The pyriform cortex has a well characterized role in regulating odor-evoked responses in rodents (Sheridan et al. 2014; Szymanski and Keller 2014). On the other hand, the posterior amygdala and posterior hypothalamus have described roles in rodents regulating stimulus mediated motor control and wakefulness, respectively (John et al. 2014; Kovac et al. 2014; Thorn-Gray and Levitt 1983). It is interesting to note that the medial orbital cortex displays widespread connections similar to the medial prefrontal cortex supporting the hypothesis that it helps regulate goal-directed behavior (Hoover and Vertes 2011). Overall, the commonality between the structures observed with increased c-Fos immunoreactivity is that they are all components of the limbic system (Fig. 12) involved in regulating memory, motivation, emotion, and olfaction. Therefore, it is not entirely surprising that some alterations in anxiety and depression-like behavior were observed in the drug injected mice. Since we have extended the existing literature surrounding TP receptors by demonstrating that peripheral antagonists directly affect neuronal activity this effect should perhaps be considered during drug development strategies involving TP receptor antagonists for treating inflammatory or thromboembolic conditions. Future studies are necessary to compare brain penetrant and non-brain penetrant TP receptor antagonists as well as the particular cell/tissue types involved in determining whether subtle changes in limbic function are altered by drug regimens.

In addition to deciphering which cell type(s) are most relevant for understanding the behavioral consequences of the TP receptor antagonist effect we observed, we appreciate that there are two forms of the TP receptor based on differential mRNA splicing of their C-terminal tails. It was originally thought that TP α was most commonly found in placental receptors, while TP β was associated with endothelial cells (Raychowdhury et al. 1994). Newer studies suggest that most tissue types, including aortic smooth muscle, umbilical vein endothelium, thymus, and small intestinal cells express both TP variants, with TP α being the more predominant form (Miggin and Kinsella 1998). The physiological implications of stimulation of each receptor subtype in varying tissues are largely unknown. Antagonism of endothelial TP α likely elicits a somewhat different response than TP β , if a drug in question binds to both forms with equal specificity or at all. Similarly, it is largely unknown how the two receptor types interact with each other, though dimerization and oligomerization has been reported (Laroche et al. 2005; Sasaki et al. 2006). Based upon the assumption that the drug did not penetrate the brain, the three days of low-dose drug treatment likely antagonized peripheral TP receptors in a both isoform and cell selective fashion to produce the changes we observed.

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Fig. 1.

Prostaglandin analysis of mouse brain tissue. Six month old male mice were ip injected with DMSO vehicle (sham) or TP receptor antagonist, SQ 29,548 at 2 mg/kg for three days. Half brain hemispheres were collected for prostaglandin extraction and quantitation by LC-MS. Pro-inflammatory prostaglandins, PGD₂ and PGE₂, were decreased in drug treated animals compared to sham injected. TXB₂, was decreased in drug treated animals compared to shams. PGF2 α and 6ketoPGF1 α levels were not altered by drug treatment. Drug treatment attenuated overall prostaglandin levels, *p<0.05, n=5.

Fig. 2.

Open field testing of TP receptor antagonist injected mice. Following three days of ip injection of DMSO vehicle (sham) or SQ 29,548 at 2 mg/kg, mice were examined by open field testing to quantify general locomotion behavior. Treatment with SQ 29,548 elicited decreases in clockwise body rotations and total body rotations as well as corner quadrant entries (Q1-4), * $p < 0.05$, $n = 13$.

Fig. 3.

Elevated zero maze testing of TP receptor antagonist injected mice. Following three days of ip injection of DMSO vehicle (sham) or SQ 29,548 at 2mg/kg, mice were examined by elevated zero maze testing to quantify anxiety-associated behavior. Drug treatment did not significantly alter time spent in open arms compared to sham injected mice, n=13.

Tail Suspension

Fig. 4.

Hanging tail suspension testing of TP receptor antagonist injected mice. Following three days of ip injection of DMSO vehicle (sham) or SQ 29,548 at 2 mg/kg, mice were examined by hanging tail suspension testing to quantify depression-associated behavior. Depression behavior was increased in SQ 29,548 treated mice compared to sham controls. Treatment with SQ 29,548 resulted in a decrease of both time spent mobile and total mobile episodes (start-stop events), * $p < 0.05$, $n = 13$.

Fig. 5.

Forced swim testing of TP receptor antagonist injected mice. Following three days of ip injection of DMSO vehicle (sham) or SQ 29,548 at 2 mg/kg, mice were examined by hanging tail suspension testing to quantify depression-associated behavior. Drug treatment did not significantly change depression behavior measured by the forced swim test. Treatment with SQ 29,548 resulted in only an increase of counterclockwise body rotations, * $p < 0.05$, $n = 13$.

Fig. 6.

Total brain PG levels significantly correlated with behavioral changes in TP receptor antagonist mice. Pearson correlation was used to measure the strength of the linear relationship between open field behavioral changes and total brain PG levels in individual mice injected three days. (A) A negative linear relationship between total prostaglandins and Q4 entries. (B) A negative linear relationship between total prostaglandins and open field max speed. (C) A positive linear relationship between total prostaglandins and tail suspension immobile episodes, $n = 5$.

Fig. 7.

The TP receptor antagonist, SQ 29,548, selectively increased c-Fos immunoreactivity. Cerebral hemispheres collected from the sham and SQ 29,548 injected mice (n=13) were fixed and serially sectioned for immunohistochemical analysis. Serial brain sections from all mice were immunostained using anti-c-Fos antibody with Vector VIP as the chromogen. A) Regions of increased c-Fos immunoreactivity included a) Medial Orbital Cortex, b) Piriform Cortex, c) Posterior Hypothalamic Area, and d) Posterior Cortical Amygdala Nucleus. B) Numbers of immunopositive nuclei per region were counted and averaged $\pm$ SD, ** $p < 0.01$, *** $p < 0.001$. C) Representative anti-c-Fos staining is shown for each region.

Fig. 8.

Pathways interconnecting elements of the limbic system neuronal interactions that include the increased c-Fos immunoreactive regions found in SQ 29,548 injected mouse brains.