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Platelets and Serotonin in Migraine

by
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Platelets and Serotonin in Migraine

University of Toledo College of Medicine

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INTRODUCTION

Migraine is a chronic disabling headache disorder of unknown etiology. There has been extensive research in the role of serotonin in the pathophysiology of migraine. Much like serotonin, migraine drugs known as triptans activate certain receptors for serotonin, 5-HT$_{1B}$ and 5-HT$_{1D}$, and promote vasoconstriction (Parsons and Whalley 1989). Serotonin also brings about vasoconstriction of cerebral arteries (Edvinsson et al. 1978). Intravenous serotonin infusions have been reported to alleviate migraine headaches in one clinical study (Kimball et al. 1960), further suggesting a role for serotonin in the circulation.

Since the platelet stores the largest amount of serotonin in the circulation (Pussard et al. 1996), the platelet has been a focus of migraine research (Malmgren and Hasselmark 1988). Although there has been considerable research in platelet serotonin storage in migraineurs during the past few decades, the results have been far from unequivocal. In studies conducted before the publication of the International Headache Society’s diagnostic criteria in 1988 (1988), there was the lack of standardized criteria for classification of headaches. The contemporary literature reveals that platelet serotonin levels in individuals with migraine may be higher (D’Andrea et al. 1994) or lower (Nakano et al. 1993) than that in unaffected individuals, but other studies (Jernej et al. 2002; Srikiatkhachorn and Anthony 1996; Joseph et al. 1989) did not report
differences between groups. Therefore, the literature is not definitive on the relationship of platelet serotonin content with migraine.

The principle aim of this project was to determine if women suffering from migraine have deficiencies of their platelet serotonin stores. There are studies that suggest serotonin is released from platelets during migraine attacks. Evidence includes decreases in platelet serotonin content and increases in serotonin metabolites in the blood during headaches (Ferrari et al. 1989), (Somerville 1976). It is known that activation of certain serotonin receptors located in the trigeminal ganglion reduce the release of a vasoactive peptide (Durham and Russo 1999). If platelets in migraine patients are deficient in serotonin, then the amount of serotonin released may not necessarily be sufficient to regulate vascular tone and pain during episodes of migraine headaches.

Given that platelets store serotonin in organelles called dense granules, dense granule deficiency in migraineurs will also be ascertained in this study. A condition with reduced numbers of platelet dense granules, δ-storage pool disorder, is associated with a bleeding diathesis (Israels et al. 1990). In a previous study, it was found that women with migraine were more likely to have bleeding diatheses, such as menorrhagia and frequent ecchymoses, than unaffected women (Tietjen et al. 2006). It is not known if there is a greater prevalence of dense granule deficiency in women with migraine.

The relationship between the quantity of platelet dense granules and their serotonin content is also unknown. Corash reasoned that there should be a
relationship between the number of dense granules and platelet serotonin content in humans (Corash et al. 1984). One study using a mouse model suggests that a reduction in the number of dense granules is associated with decreased platelet serotonin stores (Tolmachova et al. 2007). Nevertheless, there are morphological differences between mouse and human platelets (Schmitt et al. 2001). The results of this study will help elucidate the relationship, if any, between dense granule quantity and platelet serotonin content in humans.
Migraine Headache

Migraine is a chronic disorder with recurrent headaches of moderate-to-severe intensity. The International Headache Society recognizes two major types, migraine with aura and migraine without aura. Both types of migraine share a similar headache phase, which consists of a unilateral throbbing headache with a 4-to-72 hour duration accompanied by nausea, photophobia, and/or phonophobia. Furthermore, some individuals who have migraine experience an aura up to one hour before onset of headache. Auras, or subjective symptoms, are visual, sensory, or rarely, motor. Symptoms of visual aura include scintillations and scotomas. Sensory aura symptoms include paresthesias and numbness in the limbs. Auras affecting motor systems may manifest as difficulties with speech. There are no symptoms proceeding the onset of headache in migraine without aura (2004). See Table 1 for details.

Women are more likely to suffer from migraine headaches than men. In his meta-analysis, Lipton finds that the lifetime prevalence of migraine in women ranges from 7.9% to 33% in community-based studies whereas the lifetime prevalence of migraine in men ranges from 5.6% to 14.8%. The peak of migraine prevalence occurs between the ages of 25 and 55 in women (Lipton and Bigal 2005).
Table 1: Diagnostic Criteria from the International Headache Society

<table>
<thead>
<tr>
<th>Migraine without aura</th>
<th>Migraine with aura</th>
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<tr>
<td>Headache duration of 4 to 72 hours</td>
<td>Headache fulfills first three criteria for migraine without aura</td>
</tr>
<tr>
<td>Headache has two of the following characteristics:</td>
<td>At least one of the following symptoms:</td>
</tr>
<tr>
<td>Unilateral forehead pain</td>
<td>Visual: flickering light</td>
</tr>
<tr>
<td>Pulsating quality</td>
<td>(scintillation); partial to complete</td>
</tr>
<tr>
<td>Moderate to severe pain</td>
<td>loss of visual field (scotoma)</td>
</tr>
<tr>
<td>Aggravation by physical activity</td>
<td>Sensory: paresthesia,</td>
</tr>
<tr>
<td>Headache is accompanied by at least one of the following:</td>
<td>numbness</td>
</tr>
<tr>
<td>Nausea and/or vomiting</td>
<td>At least one aura symptom develops over 5 or more minutes or different</td>
</tr>
<tr>
<td>Photophobia and phonophobia</td>
<td>symptoms occur in succession over 5 or more minutes</td>
</tr>
<tr>
<td>At least 5 episodes</td>
<td>Symptoms last for 5 to 60 minutes.</td>
</tr>
<tr>
<td>Not attributed to any other disorder</td>
<td>Symptoms are unilateral.</td>
</tr>
<tr>
<td>Not attributed to any other disorder</td>
<td>At least 2 episodes</td>
</tr>
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Adapted from International Classification of Headache Disorders (2004)

*Cranial Arterial Circulation*

Cerebral arteries and their associated arterioles lie in the subarachoid space and are enveloped by pia mater. Capillaries are, however, surrounded by brain parenchyma, rather than pial cells (Zhang et al. 1990). The endothelium of brain capillaries forms the blood-brain barrier (Purves et al. 2001).

The blood supply to the meninges is separate from the blood supply to the brain. Three meningeal arteries supply blood to the meninges. These arteries are located within the dura mater.

*Models of Migraine Pathogenesis*

The cause of migraine is unknown. Several models have been proposed to explain the pathogenesis of migraine.
VASCULAR THEORY—According to the vascular theory, migraine headaches are caused by vasodilation of arteries supplying the brain and meninges. Thomas Willis first suggested this theory in the seventeenth century (Edmeads 1989). It was later popularized by Harold Wolff, an American neurologist, in the first half of the twentieth century. Wolff conducted a number of experiments to lend support to the vascular theory. In studies with migraine patients, he showed that ergotamine-induced vasoconstriction of retinal and temporal arteries coincided with relief of headache. He attributed relief of the headache to vasoconstriction and concluded that vasodilation was the cause of the pain. In contrast, Wolff also demonstrated that the aura, which could be terminated by treatment with a vasodilator, was caused by vasoconstriction (Wolff 1948). A recent study in this area showed that migraineurs have impaired endothelial-dependent vasodilatation (Schwedt 2009; Yetkin et al. 2006).

CORTICAL SPREADING DEPRESSION—Spreading depression, which Aristides Leão studied in laboratory animals by electrically, mechanically, or chemically \((K^+\)) stimulating their brains, refers to a spreading wave of depolarization followed by a reduction of electrical activity in the cerebral cortex (Leao 1944). A relationship between cortical spreading depression (CSD) and visual aura was proposed by Peter Milner, who based his findings on Karl Lashley’s descriptions of auras (Milner 1958; Lashley 1941). Evidence of CSD during auras in human subjects has been found in recent studies using functional magnetic resonance imaging and magnetoencephalography (Hadjikhani et al. 2001; Bowyer et al. 2001).
TRIGEMINOVASCULAR THEORY—According to this model, the headache phase of migraine is triggered by vasoactive peptides released from terminals of the trigeminal nerve (fifth cranial nerve) onto cranial blood vessels to induce vasodilation (Moskowitz 1984). The trigeminal nerve innervates meningeal and cerebral arteries (O'Connor and van der Kooy 1986; Edvinsson et al. 1989). Meningeal vasodilatation has been demonstrated in animals after trigeminal nerve stimulation (Messlinger et al. 1995). Cerebral vasodilatation, which is associated with an increase in blood flow in the territory of the middle cerebral artery, occurs after stimulation of the human trigeminal ganglion (Tran Dinh et al. 1992).

In both human subjects and laboratory animals, vasoactive peptides are released upon stimulation of the trigeminal ganglion (Goadsby et al. 1988). One of these vasoactive substances is calcitonin-gene related peptide (CGRP) (Messlinger et al. 1995), which is elevated in the plasma during migraine headaches (Juhasz et al. 2005). CGRP is a vasodilator encoded by the calcitonin gene. CGRP’s mechanism in vasodilation is endothelium-independent relaxation of vascular smooth muscle (Nelkin et al. 1984; Hoppener et al. 1985; Jansen-Olesen et al. 2003). Additionally, it potentiates the effect of substance P (Nyberg et al. 1988), another vasoactive peptide released by trigeminal nerve terminals (Goadsby et al. 1988). While CGRP is involved only in vasodilation, substance P plays roles in both vasodilation and nociception (Pedersen-Bjerregaard et al. 1991).
Recently, the Moskowitz group has proposed a subsequent model linking CSD and the trigeminovascular system. In rats, trigeminal denervation attenuates the increase in blood flow in the middle meningeal artery following CSD induced by electrical stimulation or pinprick (Bolay et al. 2002). Therefore, CSD may occur to trigger the aura of migraine and activate the trigeminovascular system.

**Treatment**

Prophylactic treatments include pharmacological and non-pharmacological modalities to reduce the frequency of migraine headaches. Medications, such as beta adrenergic receptor antagonists (beta blockers), tricyclic antidepressants, and anticonvulsants, have been used in prophylaxis of migraine. Examples of non-pharmacological methods include relaxation and avoidance of headache triggers (Tozer et al. 2006).

The most commonly used medications for abortive treatment of migraine headache are triptans, agonists of 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} types of serotonin receptors. They vary in lipophilicity, affinity for 5-HT\textsubscript{1B/1D} receptors, bioavailability, and half-life (Tepper et al. 2002). Varying efficacies in headache relief among triptans have also been found in meta-analyses of randomized clinical trials (Tfelt-Hansen 2006).

A major effect of triptans is vasoconstriction. 5-HT\textsubscript{1B} agonism induces vasoconstriction in isolated human meningeal and cerebral arteries (Bou et al. 2001; Razzaque et al. 2002). Treatment of experimental animals with triptans
reversed meningeal artery vasodilation induced by experimental trigeminal stimulation (Gupta et al. 2006). The inhibition of vasodilation does not occur only through 5-HT\textsubscript{1B} agonism, but also through 5-HT\textsubscript{1D} agonism. Activation of 5-HT\textsubscript{1D} at trigeminal nerve terminals indirectly leads to vasoconstriction by inhibiting release of the vasodilator, CGRP (Longmore et al. 1997; Durham and Russo 1999). In studies of human subjects during episodes of migraine headaches, administration of triptans lowered blood and salivary levels of CGRP (Juhasz et al. 2005; Bellamy et al. 2006). Therefore, triptans elicit vasoconstriction by activating 5-HT\textsubscript{1B} on the vasculature and preventing release of CGRP from trigeminal nerve terminals. The vasoconstriction occurs in meningeal arterial beds because they express 5-HT\textsubscript{1B} and CGRP receptors (Jansen-Olesen et al. 2003) and are innervated by the trigeminal nerve (O'Connor and van der Kooy 1986; Edvinsson et al. 1989).

*Pain Pathway in Migraine*

The trigeminovascular theory describes migraine as arising from activation of the trigeminal nerve (Moskowitz 1984). The activation of first order trigeminal neurons is followed by release of vasoactive peptides (Goadsby et al. 1988) and activation of second order neurons at the trigeminal nucleus pars caudalis (Storer and Goadsby 1997). Further research using animal models reveals a role for the periaqueductal gray area in trigeminal nociception because stimulation of the periaqueductal gray area inhibits the activity of second order trigeminal neurons (Knight and Goadsby 2001). Triptans may also reduce the activity of the second
order trigeminal neurons by acting on the periaqueductal gray area (Bartsch et al. 2004).

*Platelet Serotonin*

The efficacy of triptan drugs (Tfelt-Hansen 2006) and serotonin infusions (Kimball et al. 1960) in relieving migraine headache indicates that blood serotonin may play a role in affecting the course of a migraine attack. Triptans, as serotonin receptor agonists, have been shown to reduce the hyperperfusion associated with the cortical spreading depression model of migraine in rats (Gold et al. 1998). In contrast, central and peripheral serotonin depletion enhances trigeminal nucleus pars caudalis activation in response to cortical spreading depression (Supornsilpchai et al. 2006).

Platelets are useful in the evaluation of serotonin in the bloodstream because their large serotonin stores are not affected by changes in diet, unlike serotonin or its metabolites in urine and plasma (Jernej et al. 2000). A number of studies on platelet serotonin in migraineurs have been published. While some studies found no difference between migraine and control subjects' platelet serotonin (Jernej et al. 2002; Srikiatkhachorn and Anthony 1996; Joseph et al. 1989), other studies found either higher (D'Andrea et al. 1994) or lower (Nakano et al. 1993; Waldenlind et al. 1985) platelet serotonin content in subjects with migraine. Fioroni found that women with migraine during the follicular phase have more serotonin in their platelets than women without migraine in any phase.
of the menstrual cycle (Fioroni et al. 1996). In short, the relationship between migraine headache and platelet serotonin is unclear.

**Platelets**

Platelets, or thrombocytes, are small, discoid-shaped anucleate cell fragments involved in hemostasis, inflammation, and wound healing. There are 150,000 to 400,000 platelets per microliter of whole blood in the normal adult human. Platelets have organelles, including mitochondria, lysosomes, peroxisomes, and storage granules. When platelets are activated, they release the contents of lysosomes and two types of storage granules, alpha (α) and dense (δ). There are 50 to 80 α granules per platelet. α granules store adhesive glycoproteins, albumin, immunoglobulins, growth factors, platelet-specific proteins, proteoglycans, von Willebrand factor, and coagulation factors. Dense granules are less abundant than α granules. There are about 4 to 6 dense granules in each normal platelet. Their contents include adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, and ionized calcium (Lichtmann et al. 2005). Dense granules appear electron-dense because of their high calcium content; they store two-thirds of the calcium in platelets (McNicol and Israels 1999).
**Thrombocytopoiesis**

Platelets share a common origin, the pluripotent hematopoietic stem cell, with other formed elements of the blood. Pluripotent hematopoietic stem cells in the bone marrow develop into myeloid stem cells under the influence of various cytokines. Myeloid stem cells are committed to differentiation into platelets, erythrocytes, monocytes, and granulocytes (Kumar et al. 2004). A myeloid stem cell may develop into megakaryocyte high-proliferative-potential colony forming cell, the earliest megakaryocyte progenitor. This cell becomes the burst-forming unit-megakaryocyte (BFU-Mk), the earliest cell with the ability to respond to thrombopoietin (TPO). Thrombopoietin promotes proliferation of BFU-Mk and expansion of cytoplasm. As differentiation progresses, there is loss of proliferative capacity. A single BFU-Mk can undergo five to seven rounds of mitoses. In contrast, the colony-forming unit-megakaryocyte (CFU-Mk), the direct descendent of BFU-Mk, can undergo only two to five mitoses (Hoffman et al. 2004). The colony-forming unit-megakaryocyte enters stage I of megakaryocyte maturation as a megakaryoblast after a brief transitional period as a promegakaryoblast. The most salient feature of the promegakaryoblast and megakaryoblast is endomitosis, a process in which DNA is replicated without division of the nucleus (Lichtmann et al. 2005). Megakaryoblasts also develop demarcation membranes, which are thought to be sources of plasma membrane for platelet formation (Hoffman et al. 2004). At this stage, there are very few α granules. Although cultured human megakaryoblasts are capable of transporting serotonin, dense granules do not form (Yang et al. 1996). After 72 hours, the cell
enters stage II, which is characterized by increases in the number of storage granules as well as cell volume. Platelet release occurs in stage III and IV megakaryocytes. One megakaryocyte fragments into 2000 to 3000 platelets with bending of long, thin extensions also known as proplatelets. Remaining cell debris is removed by macrophages (Hoffman et al. 2004).

The normal adult human produces one hundred billion platelets per day to maintain a stable number of platelets in circulation. Platelet production, regulated by TPO, increases when there is thrombocytopenia or inflammation. In these conditions, TPO levels in blood and bone marrow tend to increase. Thrombopoietin is constitutively synthesized in liver and kidney; its synthesis can be induced in spleen and bone marrow. Thrombopoietin levels are regulated by synthesis and degradation. In the reactive thrombocytosis of inflammation, a cytokine, interleukin-6, increases hepatic TPO synthesis. Its synthesis is also upregulated in response to thrombocytopenia. Platelets also contribute to the regulation of TPO by receptor-mediated uptake and degradation through Mpl, the receptor for TPO. According to the model proposed by Kaushansky, platelets remove TPO from circulation to reduce the megakaryocyte-proliferating effects when the size of the platelet population is within normal limits (Kaushansky 2005).

Platelet Aging

Newly released platelets tend to be larger than older platelets although newly released platelets are not uniform in size (Corash et al. 1984). Platelet
density depends on the number of \( \alpha \) granules in the platelet. Density decreases as platelets age because activation results in losses of \( \alpha \) granules (van Oost et al. 1984). Further evidence supporting the association between platelet size and age is the high RNA content of large platelets (Rinder et al. 1998). In conditions in which thrombopoiesis is upregulated, the population of large platelets with elevated RNA content expands (Kienast and Schmitz 1990). Large platelets have more dense granules than small platelets. The number of dense granules decreases with activation. However, the number of mitochondria is not related to platelet size because mitochondria are not released from activated platelets (Corash et al. 1984). Mitochondrial enzyme activity, except for the outer mitochondrial membrane-bound monoamine oxidase B (MAOB), does not change with age. MAOB activity is lower in smaller platelets (Murphy et al. 1978).

Platelets persist in the circulation for up to ten days (Harker 1977). Senescent platelets are cleared from the circulation by the reticuloendothelial system in the spleen (Klonizakis et al. 1981). It has been suggested the loss of plasma membrane phospholipid asymmetry triggers the spleen to destroy platelets (Pereira et al. 2002).

**Dense Granule Calcium, Adenine Nucleotides, and Serotonin in Hemostasis**

The adenine nucleotide, adenosine diphosphate (ADP), triggers platelet aggregation through activation of purinergic receptors, P2Y. ADP by itself is considered to be a weak trigger for aggregation, but it acts synergistically with
thrombin and collagen. Another adenine nucleotide in the dense granule, adenosine triphosphate (ATP), activates P2X receptors. P2X activation results in an influx of calcium into the platelet (Packham and Mustard 2005).

Aside from a calcium requirement for activation of factors V and IX in the coagulation cascade, calcium plays a part in platelet activation. However, the relative contributions of calcium stores to platelet activation from dense granules and the dense tubular system (endoplasmic reticulum remnants) are not known (Varga-Szabo et al. 2009).

The exact role of platelet-derived serotonin in hemostasis has not been identified. Serotonin is likely to play some role in hemostasis. In humans, platelet serotonin depletion with use of SSRIs is associated with increased bleeding, including perioperative and gastrointestinal bleeding (Gartner et al. 2010), (Dall et al. 2009). There is further evidence for a role for serotonin in hemostasis in animal models. Platelet dense granule serotonin secretion increases alpha-granule secretion through serotonylation, or modification of enzymes by serotonin (Walther et al. 2003). Serotonylation also occurs during the formation of collagen- and thrombin-activated (COAT) platelets, a platelet subpopulation with retained alpha-granule proteins on the surface that may have enhanced procoagulant properties (Dale 2005).

**Calcium in Platelet Dense Granules**

Dense granules contain large amounts of ionic calcium. Holmsen, et al. determined the concentration to be 2 molar. Some calcium must be trapped in
insoluble complexes as the calcium concentration in the dense granule is beyond the solubility of calcium (Holmsen and Weiss 1979).

Calcium enters platelets in a mechanism called store-mediated calcium entry (Nakamura et al. 1995) because platelets lack voltage-gated calcium channels (Pannocchia et al. 1987). Store-mediated calcium entry is facilitated by depletion of two intracellular calcium stores (Rosado et al. 2004), which are in the dense tubular system as well as the dense granule (Lichtmann et al. 2005). Calcium release from the dense tubular system and/or the dense granule triggers reorganization of the actin cytoskeleton. Then pp60src, which is associated with the actin cytoskeleton, activates Bruton’s tyrosine kinase. Bruton’s tyrosine kinase is directly or indirectly responsible for store-mediated calcium entry (Redondo et al. 2005).

Lopez and colleagues have studied calcium transport in calcium-storing lysosome-related acidic organelles in platelets (Lopez et al. 2005). Although they have not verified that the lysosome-related organelles are indeed dense granules, these organelles share many similarities with dense granules. Like dense granules, these organelles release calcium when the platelet is stimulated with thrombin (Ruiz et al. 2004; Lopez et al. 2005). Dense granules are slightly acidic with a pH of 6 (Holmsen and Weiss 1979). They also express a lysosome-related surface marker, lysosome associated membrane protein-2 (Israels et al. 1996). To enter the organelle identified by Lopez, Ca\(^{2+}\) is actively transported by a Ca\(^{2+}\) ATPase as long as the H\(^+\) ATPase maintains the acidity of the dense granule (Lopez et al. 2005).
Adenine Nucleotides in Dense Granules

Dense granules store two-thirds of all adenine nucleotides, primarily adenosine diphosphate (ADP) and adenosine triphosphate (ATP), in the platelet. ATP and ADP forms insoluble aggregates with calcium. There is about two-thirds as much ADP as ATP in the dense granule. This pool of adenine nucleotides is not utilized in the platelet’s metabolic activities because it does not exchange with adenine nucleotides in the cytosol (Holmsen 1985).

The adenine nucleotides in the dense granule are synthesized from adenine in the platelet. Adenine is not produced in the platelet; it is transported from outside the cell by a specific transporter. Then adenine enters the purine salvage pathway by which it is converted to adenosine monophosphate (AMP) with phosphoribosyl pyrophosphate and adenine phosphoribosyltransferase. Phosphorylation of AMP to ADP and ATP is catalyzed by adenylate kinase (Holmsen 1985).

Multidrug resistance protein-4 (MRP4), a member of the ATP binding cassette superfamily, is the putative adenine nucleotide transporter on the dense granule membrane. MRP4 and MRP5 are the two known nucleotide transporters in mammalian cells, but MRP5 is not found in platelets. MRP4 has the highest expression in the dense granule fraction among other platelet organelle and membrane fractions. It can transport ADP, cAMP, cGMP, and possibly other nucleotides (Jedlitschky et al. 2004).
Serotonin in Platelet Dense Granules

Platelets take up serotonin from plasma through a membrane-bound serotonin transporter (SERT). The serotonin transporter is a sodium-dependent serotonin channel with twelve putative transmembrane domains. In the current model for the mechanism of serotonin transport, the binding of sodium and serotonin, followed by chloride, to the outside of the transporter causes a conformational change in the protein. Potassium binds to the transporter’s intracellular part to restore the transporter to its active conformation after sodium, chloride, and serotonin are released inside the platelet (Ni and Watts 2006). Cell surface SERT expression is regulated by glycosylation and phosphorylation. N-linked glycosylation of asparagine residues allows SERT to fold properly for cell surface expression. However, it does not play a role in ligand selectivity (Blakely et al. 1994). A basal level of phosphorylation mediated by a mitogen-activated protein kinase (MAPK) also promotes cell surface SERT expression. In contrast, activated protein kinase C phosphorylates SERT at other amino acid residues to cause SERT to be internalized to an intracellular pool (Samuvel et al. 2005).

Serotonin enters the platelet dense granule through the reserpine- and tetrabenazine-sensitive vesicular monoamine transporter 2 (VMAT2) by secondary active transport. VMAT2 transfers serotonin into the dense granule and expels protons into the cytosol. Dense granule serotonin storage levels regulate VMAT2 activity. Increased amounts of serotonin in dense granules reduce serotonin uptake (Holtje et al. 2003).
Cyclic guanosine monophosphate (cGMP) phosphodiesterase inhibitors and selective serotonin reuptake inhibitors (SSRIs) alter SERT-mediated serotonin transport through secondary messenger systems. cGMP phosphodiesterase inhibitors increase serotonin transport. They activate protein kinase G, which in turn activates MAPK, resulting in an increase in the number of serotonin transporters on the cell surface (Prasad et al. 2005; Zhu et al. 2004). SSRIs reduce serotonin transport and deplete platelet serotonin stores (Hergovich et al. 2000). In computer-generated models, SSRIs are predicted to close the pore for serotonin after binding to SERT (Ravna et al. 2006). The direct inhibition of serotonin transport is responsible for reduced serotonin flux during acute SSRI treatment. However, in long-term treatment, there is reduced cell surface SERT expression without change in the amount of mRNA, which means SSRIs reduce serotonin transport in chronic treatment by affecting secondary messenger systems (Benmansour et al. 2002).

Secretion of Serotonin

Serotonin can be released from resting platelets via SERT-mediated outward transport. In studies of intact platelets, efflux is induced by elevation of cytosolic calcium or sodium concentrations (Turetta et al. 2004). Alteration of internal ionic conditions must cause the transporter to switch from the outward facing to the inward facing conformation according to the alternate access model (Korkhov et al. 2006). Nevertheless, in SERT-tranfected cells, the rate of influx is greater than the rate of efflux (Sitte et al. 2001). Activation of PKCε also
promotes SERT-mediated serotonin efflux. Amphetamines are known to activate PKC and increase serotonin efflux (Seidel et al. 2005), but it is not known if there are any conditions that activate PKCε in platelets. Before SERT-mediated efflux can occur, serotonin must be released from its storage vesicle. In cultured cells, amphetamines along with reversal of the pH gradient cause VMAT2 to transport amines outwardly. This effect has been demonstrated with dopamine, but not with serotonin (Wilhelm et al. 2006).

Larger amounts of serotonin are released from activated platelets than from SERT-mediated efflux. Before platelets are activated, they adhere to endothelium and aggregate with each other in preparation for thrombus formation with the occurrence of endothelial injury, including blood vessel breakage and atherosclerotic plaque rupture. Activation and adhesion are triggered by endothelial injury with exposure of the subendothelial extracellular matrix components, including collagen and von Willebrand factor (vWF), to the lumen of the blood vessel. Platelet vWF receptor GPIb-IX binds to subendothelial vWF to weakly adhere the platelet to the site of endothelial injury. The platelet’s shape changes from the discoid shape of the resting platelet to a stellate shape. After vWF interacts with GPIb/IX, collagen binds to the GPIb/IX/V complex, integrin αIIbβ3 (GpIIb/IIIa), and collagen receptor α2β1 (Gpla/IIa) to induce platelet activation and strong adhesion through a signal transduction cascade inside the platelet. This leads to release of ADP and thromboxane A₂ (TxA₂), and activation of αIIbβ3. Increased surface expression of αIIbβ3 is a characteristic of platelet activation. Thrombin, TxA₂, ADP, and collagen activate platelets by activating
phospholipase C\(\beta\), which increases platelet cytosolic calcium concentration and activates protein kinase C (PKC). Formation of a contractile ring around the storage granules requires interaction between actin and myosin, and elevated intracellular calcium concentration. PKC plays a role in fusion of the dense granule with the platelet plasma membrane (Hoffman et al. 2004).

Dense granule docking and fusion are requisites for release of serotonin from platelets by exocytosis. In the formation of the first docking complex, called 7S, soluble N-ethylmaleimide-sensitive attachment protein receptors (SNAREs) on the granule and the inner surface of the platelet plasma membrane recognize each other. This is followed by formation of a 20S docking complex involving N-ethylmaleimide sensitive factor (NSF), synaptosomal-associated proteins (SNAPs), and other chaperone proteins. PKC phosphorylates SNAPs. During fusion, SNAPs and NSF interact to activate the ATPase domain of NSF. The result is disruption of the 20S docking complex. Finally, exocytosis of dense granule contents, including serotonin, occurs with increases in local calcium concentration and pore formation (Flaumenhaft 2003; McNicol and Israels 1999).

**Serotonin**

Serotonin, 5-hydroxytryptamine, or 3-(β-aminoethyl)-5-hydroxyindole was identified in the 1950s by Irvine H. Page as a substance released from platelets...
during clot formation (Page 1952). In 1952, Vittorio Erspamer concluded that serotonin was the same compound as enteramine, which he had earlier localized to gastrointestinal tissues (Erspamer and Asero 1952). In 1953, Page found serotonin in brain (Twarog and Page 1953).

*Biosynthesis and Metabolism*

Serotonin biosynthesis occurs in two steps. In the first step, tryptophan hydroxylase converts the amino acid L-tryptophan to L-hydroxytryptophan. In the second of the two steps, L-hydroxytryptophan is converted to serotonin by aromatic L-amino acid decarboxylase (Grahame-Smith 1967).

In the central nervous system (CNS), serotonin is primarily synthesized in the dorsal raphe nucleus of the medulla oblongata (Cohen et al. 1996). The precursor to serotonin in the CNS is L-tryptophan, which crosses the blood-brain barrier via the large neutral amino acid transporter type 1 (Boado et al. 2003). After serotonin is synthesized in the neuron cell bodies, it is packaged into membrane-bound vesicles. The vesicles are transported down axons for release from axon terminals. Serotonergic neurons, whose cell bodies are in the dorsal raphe nucleus, project axons to other areas of the brain (Purves et al. 2001).

The blood-brain barrier separates serotonin in peripheral circulation from that in the brain and spinal cord, or the central nervous system. Lipophilic and gaseous molecules can freely diffuse across brain capillary endothelial cells that form the blood-brain barrier, but hydrophilic substances only pass through the blood-brain barrier when specific transporters are present (Purves et al. 2001).
Although brain capillary endothelial cells in the mouse have been found to express serotonin transporters on luminal and abluminal sides (Wakayama et al. 2002), monoamine oxidase A degrades serotonin inside the endothelial cells to prevent transport of serotonin across the blood-brain barrier (Yu 1984).

Outside of the central nervous system, serotonin is synthesized primarily by enterochromaffin cells in the crypts of Lieberkühn in the small intestine. Eighty percent of the serotonin synthesized in the enterochromaffin cells is retained in the cells. Release of serotonin into the portal circulation from enterochromaffin cells is facilitated by stimulation of muscarinic acetylcholine and β-adrenergic receptors, increased intestinal luminal pressure, and decreased luminal pH. Histamine, GABA, adenosine, and somatostatin inhibit release of serotonin (Kema et al. 2000; Racke et al. 1996). Platelets sequester ninety-five percent of the serotonin in blood (Pussard et al. 1996).

There are two routes for catabolism of serotonin. One pathway involves oxidation. The first step is the oxidative deamination of serotonin to 5-hydroxyindole acetaldehyde, which is catalyzed by monoamine oxidase (MAO). MAO is found in the liver and lungs as well as in the brain. Outside of the brain, the liver is responsible for first-pass metabolism of serotonin, which reaches the liver by the portal vein. Although both A and B isoforms of monoamine oxidase are capable of catalyzing the oxidation of serotonin, monoamine oxidase A (MAOA) has higher affinity for serotonin than monoamine oxidase B (MAOB). The next and last step is conversion of 5-hydroxyindole acetaldehyde to 5-hydroxyindole acetic acid (5-HIAA) by aldehyde dehydrogenase. 5-
Hydroxyindole acetaldehyde is excreted in urine. A small amount of serotonin is also excreted unchanged in urine (Brunton et al. 2005). Another pathway for removal of serotonin is with synthesis of melatonin. In the pineal gland and, to a smaller extent, in liver, lymphocytes, platelets, and retina, serotonin is utilized in the synthesis of melatonin (Pandi-Perumal et al. 2006).

Measurement of Serotonin

High performance liquid chromatography (HPLC) has been routinely used to measure the amount of serotonin in specimens, such as blood, urine, and cerebrospinal fluid. The principle behind HPLC is separation of complex mixture of different compounds by differential retention time in a solid column. High performance liquid chromatography systems consist of mobile and stationary phases, and a detector. The mobile phase contains solvents to carry the sample to the stationary phase, or column, and subsequently, the detector (Kema et al. 2000). As the sample carried in the mobile phase flows through the column, the analytes in the sample are deposited on the surfaces of the column’s silica particles. The pore size is controlled by chemical modification of the silica particle surface. In HPLC of serotonin, octyldodecyl (C$_{18}$)-modified silica with a pore size of 100 Å is used. Small pores reduce the adsorption of larger molecules from the sample onto the silica beads. The retention time, which is based on the analyte’s elution speed, depends on the analyte’s non-covalent interactions with the stationary phase (Katz et al. 1998). The composition of the mobile phase also affects the retention time of serotonin (Morgadinho et al.
The serotonin-containing sample is eluted into a fluorometric or electrochemical detector. Fluorometric detection is based on the 300-to-370 nm emission spectra of serotonin when excited in the range of 280 to 320 nm. In electrochemical detection, a small electrical current discharged during oxidation of serotonin is measured. For serotonin, electrochemical detection is considered more sensitive than fluorometric detection (Kema et al. 2000).

Serotonin is unstable in tissue samples. It is oxidized by superoxide anion radicals (Wrona and Dryhurst 1998), denatured oxyhemoglobin (Blum and Ling 1959), and in some tissues, monoamine oxidases (Kema et al. 2000). Unfortunately, the deproteination step in serotonin extraction for HPLC denatures hemoglobin. Oxidation of serotonin in blood samples can be prevented completely by converting oxyhemoglobin to carboxyhemoglobin with carbon monoxide. Another method is the use of ascorbate as an antioxidant (Blum and Ling 1959). However, ascorbate is not compatible with electrochemical detection in HPLC because electrical potential changes from oxidation of ascorbate interfere with detection of current induced by oxidation of serotonin (Kema et al. 2000).

An alternative to HPLC is enzyme-linked immunosorbent assay (ELISA). Antibodies are used in this method to detect and quantify an antigen, such as a protein, peptide, or smaller molecule. One type, the competitive ELISA, is used for detection of small antigens, such as serotonin, that have only epitope for antibody recognition. An aqueous sample and a solution with detection antibodies recognizing the antigen of interest are added to the test plate with
covalently bound antigen. The plate-bound antigen competes with an unknown amount of antigen in the sample for binding to the detection antibody. The detection antibody binds to antigen in solution and antigen bound to the plate. Antigen-antibody complexes in solution are washed away, but antibodies on the bound antigen remain on the plate. The remaining antibody present on the plate is inversely related to the amount of antigen in the sample. In the direct detection method, the detection antibody carries a chemically coupled enzyme, such as horseradish peroxidase or alkaline phosphatase. In the indirect detection method, a secondary antibody, recognizing the fragment of crystallization (Fc) region of the detection antibody, is chemically coupled with the enzyme. Indirect detection has the advantage of signal amplification and the disadvantage of increased probability of nonspecific binding. In both methods, the chemically coupled enzyme generates a colored complex when a substrate is added. A spectrophotometer is used to measure color intensity. The absolute quantity of serotonin present in the sample is calculated from a standard curve (Crowther 2000).

Flow cytometry has been used to detect serotonin in platelets, but this method is not quantitative. In flow cytometry, fluorophore-labeled antibodies are used to detect serotonin in permeabilized fixed platelets. The flow cytometer measures the amount of fluorescence emitted from individual cells. Unlike ELISA, which permits absolute quantification of antigen in a population of platelets, flow cytometry data describes proportions of cells exceeding a threshold level of fluorescence (Gobbi et al. 2003; Maurer-Spurej et al. 2007).
Serotonin has divergent roles in regulating physiological functions due to existence of different receptor subtypes. In central nervous system, serotonin acts as a neurotransmitter. Outside of the central nervous system, serotonin has been implicated in the control of smooth muscle tone, platelet aggregation, and cell proliferation. Serotonin receptors are found in many different tissues. The various serotonin receptors are listed in Table 1.

Table 1: Serotonin Receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue Distribution</th>
<th>Signal Transduction Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁₅</td>
<td>5-HT₁&lt;sup&gt;₁&lt;/sup&gt; Human: cerebral cortex, hippocampus, raphe nucleus, spinal cord, coronary artery, heart atrium, kidney&lt;sup&gt;₁⁻⁴&lt;/sup&gt; Rat: hippocampus, medulla, raphe nucleus, septum, dorsal root ganglion, sympathetic ganglion, kidney&lt;sup&gt;⁴⁻⁶&lt;/sup&gt; Mouse: macrophage&lt;sup&gt;⁷&lt;/sup&gt;</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;: inhibit adenylyl cyclase; activate PLC&lt;sup&gt;⁴⁷&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-HT₁₆</td>
<td>Human: basal ganglia, hippocampus, substantia nigra, trigeminal ganglion, endothelium, vascular smooth muscle&lt;sup&gt;⁸⁻¹⁰&lt;/sup&gt; Rat: cerebellum, basal ganglia, substantia nigra, dorsal root ganglion, sympathetic ganglion, trigeminal ganglion, epididymis, heart valve, lymphocyte&lt;sup&gt;⁵,¹¹⁻¹⁵&lt;/sup&gt; Mouse: hypothalamus&lt;sup&gt;¹⁶&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5-HT₁₇</td>
<td>Human: basal ganglia, cerebral cortex, trigeminal ganglion, spinal cord&lt;sup&gt;⁸,¹⁰&lt;/sup&gt; Rat: dorsal root ganglion, sympathetic ganglion, trigeminal ganglion&lt;sup&gt;⁵,¹³&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5-HT₁₈</td>
<td>Human: amygdala, basal ganglia, cerebral cortex&lt;sup&gt;¹⁷,¹⁸&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
| 5-HT<sub>1F</sub> | Human: coronary artery, cranial artery, heart atrium, heart ventricle<sup>3</sup>  
Rat: dorsal root ganglion, sympathetic ganglion, trigeminal ganglion, lymphocyte<sup>5,13,15</sup> |  |
| 5-HT<sub>2</sub> |  | G<sub>q</sub>: activate PLC<sup>47</sup> |
| 5-HT<sub>2A</sub> | Human: basal ganglia, cerebral cortex, hippocampus, spinal cord, heart atrium, platelet, vascular smooth muscle<sup>3,19,20</sup>  
Rat: brain (most areas), dorsal root ganglion, sciatic nerve, spinal cord, sympathetic ganglion, aorta, lymphocyte, renal artery<sup>5,15,21-23</sup> |  |
| 5-HT<sub>2B</sub> | Human: endothelium, kidney, liver, pancreas, small intestine, spleen, trachea, uterus, vascular smooth muscle<sup>9,24,25</sup>  
Rat: artery, epididymis, heart valve, lymphocyte, vein<sup>11,12,15,23,26,27</sup>  
Mouse: cerebellum, bronchial smooth muscle, gastrointestinal tract smooth muscle, myocardium<sup>28</sup> | G<sub>q</sub>: activate PLA<sub>2</sub> & PLC, couple to nitric oxide synthases<sup>47,48</sup> |
| 5-HT<sub>2C</sub> | Human: amygdala, basal ganglia, cerebellum, cerebral cortex, choroid plexus, hippocampus, hypothalamus, olfactory tubercle, substantia nigra<sup>29,30</sup>  
Rat: brain (most areas), dorsal root ganglion<sup>5,31</sup> | G<sub>q</sub>: activate PLC<sup>47</sup> |
| 5-HT<sub>3</sub> | Human: area postrema, dorsal vagal motor nucleus, nucleus tractus solitarius, myenteric plexus, trigeminal ganglion, lymphocyte<sup>32,33</sup>  
Rat: cerebral cortex, hippocampus, dorsal root ganglion, sympathetic ganglion, lymphocyte<sup>5,34</sup> | Ligand-gated ion channel<sup>47</sup> |
| 5-HT<sub>4</sub> | Human: basal ganglia, cerebral cortex, hippocampus, endothelium, heart, myenteric plexus<sup>9,39</sup>  
Rat: amygdala, basal ganglia, hippocampus, olfactory tubercle, septum<sup>36</sup>  
Mouse: amygdala, basal ganglia, hippocampus, olfactory tubercle, septum<sup>37</sup> | G<sub>5</sub>: activate adenylyl cyclase<sup>47</sup> |
| 5-HT<sub>5</sub> |  |  |
### 5-HT5A
- **Human:** amygdala, basal ganglia, cerebellum, hypothalamus, substantia nigra, thalamus, lymphocyte
- **Rat:** cerebellum, cerebral cortex, hippocampus, hypothalamus, pons, thalamus, spinal cord, carotid body, sympathetic ganglion

| G<sub>i</sub>: inhibit ADP ribosyl cyclase & adenylyl cyclase<sup>47</sup> |

### 5-HT5B
- **Rat:** hippocampus<sup>40</sup>
- **Mouse:** hippocampus, raphe nucleus<sup>42</sup>

### 5-HT6
- **Human:** basal ganglia, cerebral cortex, raphe nucleus, thalamus<sup>43</sup>
- **Rat:** basal ganglia, cerebellum, cerebral cortex, hippocampus, dorsal root ganglion, sympathetic ganglion, lymphocyte<sup>15,44</sup>

| G<sub>q</sub>: activate PLC<sup>47</sup> |

### 5-HT7
- **Human:** basal ganglia, hippocampus, raphe nucleus, thalamus, trigeminal ganglion, GI tract, vascular smooth muscle, ventricle wall<sup>45-47</sup>
- **Rat:** amygdala, basal ganglia, cerebral cortex, hippocampus, hypothalamus, dorsal root ganglion, sympathetic ganglion, lymphocyte<sup>46</sup>
- **Mouse:** hypothalamus<sup>16</sup>

| G<sub>q</sub>: activate PLC<sup>47</sup> |

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**PLA2, phospholipase A2; PLC, phospholipase C**


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**Signal Transduction Mechanisms of Serotonin Receptors**

All serotonin receptors, except 5-HT<sub>3</sub>, are G protein coupled receptors (GPCRs). The G protein is associated with the intracellular segment of the receptor. When a ligand binds to a GPCR, the α subunit of the G protein exchanges its bound guanosine diphosphate (GDP) for guanosine triphosphate.
(GTP). This exchange triggers the α subunit to disconnect from β and γ subunits. The α subunit reunites with βγ subunits after the intrinsic GTPase activity of the α subunit catalyzes the hydrolysis of GTP to GDP. The G protein is physiologically active when the α subunit is separated from β and γ subunits (Alberts et al. 2002).

Figure 1. G Protein Coupled Receptors. Upon activation of a G protein-coupled receptor, the G protein α subunit dissociates from β and γ subunits. Different forms of G protein are composed of subunits with different functions. (A) When a receptor is coupled to a stimulatory G protein, or Gs, receptor activation triggers the α subunit to bind to adenylyl cyclase and activate it. Adenylyl cyclase catalyzes the formation of cAMP from ATP. cAMP activates PKA. (B) When a receptor is coupled to an inhibitory G protein, or Gi, receptor activation triggers the distinct α subunit of Gi to inhibit adenylyl cyclase. (C) When a receptor coupled to Gq is activated, PLC is stimulated. PIP2 in the cell membrane is cleaved into IP3 and DAG by PLC. In one pathway, DAG activates PKC. In the other pathway, IP3 triggers the release of calcium ions into the cytosol by opening calcium channels in the endoplasmic reticulum. The calcium is free to bind to any calcium binding proteins in the cytosol. DAG may also be converted to arachidonic acid. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP3, inositol triphosphate; PIP2, phosphatidylinositol bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C. (Based on Alberts et al. 2002)
Metabotropic serotonin receptors are coupled to $G_s$, $G_i$, or $G_q$ forms of G protein (see Figure 1). Receptors belonging to the 5-HT$_1$ and 5-HT$_5$ subfamilies are coupled to an inhibitory G protein ($G_i$); activation of these receptors leads to inhibition of adenylyl cyclase and decrease of cyclic adenosine monophosphate (cAMP) levels. 5-HT$_1$ receptors may also activate phospholipases. 5-HT$_4$ is coupled to a stimulatory G protein ($G_s$), which activates adenylyl cyclase to increase cAMP and activate protein kinase A. 5-HT$_2$, 5-HT$_6$, and 5-HT$_7$ types of serotonin receptors couple to $G_q$. $G_q$ activates phospholipase C. This pathway leads to activation of PKC and an increase in cytosolic calcium levels. One member of the 5-HT$_2$ subfamily, 5-HT$_{2B}$, activates phospholipase $A_2$ (PLA$_2$) (Noda et al. 2004). PLA$_2$ converts plasma membrane phospholipids to arachidonic acid, a precursor to biologically active compounds known as eicosanoids (Brunton et al. 2005).

Regulation of Vascular Tone

Serotonin modulates vascular tone through activation of its receptors. Although serotonin was discovered as a vasoconstrictor released from platelets during clotting (Page 1952), it may act as a vasoconstrictor or a vasodilator depending on the type of receptor with which it interacts.

Vasoconstriction requires activation of 5-HT$_{1B}$ (also called 5-HT$_{1Dβ}$) receptors, which are expressed on endothelial and vascular smooth muscle cells.
In cultured cells ectopically expressing the human 5-HT$_{1B}$, receptor activation with an agonist, such as sumatriptan, increases the concentration of inositol triphosphate (IP$_3$) and decreases the concentration of cAMP in the cytosol. IP$_3$ is responsible for increasing the calcium concentration in the cytosol, which triggers smooth muscle contraction (Dickenson and Hill 1998).

5-HT$_{2B}$ is another serotonin receptor expressed on endothelial and vascular smooth muscle cells. Although 5-HT$_{2B}$ couples to G$_q$ and elicits IP$_3$ production much like 5-HT$_{1B}$, activation of 5-HT$_{2B}$ is associated with vasodilation, rather than vasoconstriction. 5-HT$_{2B}$ differs from 5-HT$_{1B}$ in that the C terminus of 5-HT$_{2B}$ recruits nitric oxide synthase (NOS) to promote production of nitric oxide (NO). Endothelium expresses inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) (Manivet et al. 2000), and vascular smooth muscle expresses all three NOS isoforms, including neuronal NOS (nNOS or NOS-1), iNOS, and eNOS (Buchwalow et al. 2002). Coupling of 5-HT$_{2B}$ and NOS have been observed in studies with cultured cells and animals (Manivet et al. 2000; Ellwood and Curtis 1997). NO is the vasodilator produced upon 5-HT$_{2B}$ activation (Manivet et al. 2000). NO produced in both endothelium and vascular smooth muscle causes vascular smooth muscle to relax, which promotes vasodilation (Buchwalow et al. 2002). NOS generates NO from L-arginine and molecular oxygen. NO is a small gaseous molecule that readily diffuses across cell membranes. Once NO enters the vascular smooth muscle cell, NO activates a soluble guanylyl cyclase (sGC). sGC catalyzes the formation of cyclic
guanosine monophosphate (cGMP) from guanosine triphosphate. cGMP relaxes vascular smooth muscle (Garcia and Stein 2006).

**Serotonin Receptors and Migraine**

In sum, the key serotonin receptor implicated in migraine is 5-HT$_{1B}$, where triptan and ergot drugs act as agonists. At this receptor, triptans, ergot derivatives, and serotonin act as vasoconstrictors. Vasoconstriction can be elicited by these substances in both cranial and extracranial vascular beds although vasoconstriction of cranial vasculature is more relevant for symptomatic relief of migraine headaches (Silva et al. 2007).
Subjects and Blood Collection

61 women with migraine were recruited from a headache clinic. 42 women without history of any headache disorder were recruited from a general medicine clinic to serve as control subjects. Inclusion criteria for both migraine and groups include women aged 18 to 64 years and written consent. Subjects were placed in the migraine group if the characteristics of their headaches met the criteria of the International Classification of Headache Disorders for migraine without aura or migraine with aura. Control subjects did not have headaches that met criteria for any type of migraine, probable migraine, or other headache disorder defined in the International Classification of Headache Disorders. Exclusion criteria for both groups are pregnancy and active malignancy. All subjects completed a survey assessing bleeding, migraine, and other health history. 40 out of 101 questions on the survey addressed bleeding and menstrual histories. A pictorial chart described by Janssen et al. was used to assess menstrual blood loss (see Figure 2), where a score of 185 or greater defines menorrhagia (Janssen et al. 1995). Subjects completed the pictorial chart based on retrospective patterns of typical menstrual pad and tampon usage. Another 20 questions addressed conditions such as hypertension, diabetes mellitus, vascular disease, stroke, heart disease, thrombosis, arthritides, endometriosis, and neurologic and psychiatric disorders. The remainder of the questions focused on migraine symptoms and severity. These questions were based on criteria from the
International Classification of Headache Disorders (2004). 8.5 ml blood was collected by venipuncture from the antecubital vein in Vacutainer tubes with acid citrate dextrose anticoagulant (Becton Dickinson, Franklin Lakes, NJ). The protocol was approved by the Institutional Review Board at the University of Toledo Health Sciences Campus.

Figure 2. **Pictorial Chart for Assessment of Menstrual Blood Loss.** This chart is based on a chart that appears in Janssen’s 1995 paper.
Materials

All chemicals were obtained from Sigma (St. Louis, MO), except for phosphoenolpyruvate (PEP) and pyruvate kinase (PK), which were from Roche Diagnostics (Indianapolis, IN). ATP Assay Mix and ATP Assay Mix Dilution Buffer were also from Sigma. The Serotonin EIA kit was obtained from Rocky Mountain Diagnostics (Colorado Springs, CO).

Blood Processing

Blood was processed within 24 hours of venipuncture. Blood tubes were centrifuged in a Helmer Universal 32 centrifuge (Helmer, Noblesville, IN) at 375g for 10 minutes at room temperature. Platelet-rich plasma (PRP) was removed and placed into polypropylene tubes.

Platelet Enumeration

A Coulter Gen S Hematology Analyzer (Beckman Coulter, Fullerton CA) was used according to manufacturer’s instructions for determination of number of platelets in PRP.

Electron Microscopy

Whole Mount Preparation: Carbon- and parlloidin-coated copper grids (Electron Microscopy Sciences, Fort Washington, PA) were incubated with 10 µl PRP
drops on paraffin film for 10 minutes. The grids were subsequently washed three times with deionized water and allowed to air-dry.

Dense Granule Enumeration: Platelets were visualized using a Philips TM10 electron microscope (Philips, Eindhoven, Netherlands). Dense granules were enumerated from 100 platelets for each subject.

Platelet Serotonin Studies

200 µl PRP was diluted with 800 µl phosphate-buffered saline and centrifuged at 4500g for 10 minutes. The supernatant was discarded. The platelet pellet was resuspended in 200 µl of 5 mM ascorbic acid. Samples were stored at -30° C.

Serotonin in platelets was determined by enzyme-linked immunosorbent assay with a Serotonin EIA kit. Samples were thawed at room temperature. When completely thawed, they were centrifuged at 6500g for 2 minutes. For the serotonin acylation reaction, 10 µl of the supernatant was incubated with 25 µl Acylation Reaction and 250 µl Acylation Buffer for 15 minutes at room temperature on an orbital shaker set at 500 rpm. Then 10 µl of the acylated supernatant was incubated with 50 µl anti-serotonin primary antibody in a plate from the Serotonin EIA kit for one hour at room temperature on an orbital shaker set at 500 rpm. The plate was washed twice with the Wash Buffer using a MutiWash Advantage plate washer (Tricontinental, Suffolk, United Kingdom). 100 µl Enzyme Conjugate, the secondary antibody, was added to the plate and incubated at room temperature on an orbital shaker set at 500 rpm for 30 minutes. After this incubation, the plate was washed three times with the Wash
Buffer using a plate washer. 100 µl Substrate was added to the plate and incubated for 25 minutes at room temperature on an orbital shaker set at 500 rpm. Then 100 µl Stop Solution was added to the plate. Absorbance was read at 450 nm on a Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Samples were prepared in duplicate. A standard curve was prepared along side the supernatants using six Serotonin Standards included in the Serotonin EIA kit. The serotonin concentration in the standards range from 0 ng/ml to 2500 ng/ml. The concentration of serotonin in the samples was calculated from the standard curve using Softmax Pro software (Molecular Devices, Sunnyvale, CA).

Adenine Nucleotide Assay

A bioluminescent method was used to determine content of adenine 5’-triphosphate (ATP) and adenine 5’-disphosphate (ADP). 450 µl 96% ethanol and 50 µl 0.1 M EDTA was added to 500 µl PRP. This extract was stored at -30º C. ATP Assay Mix and ATP Assay Mix Dilution Buffer were dissolved according to manufacturer’s instructions. A working mix was prepared with 24 ml ATP Assay Mix Dilution Buffer and 25 ml HEPES buffer containing 5 mM pH 7.8 HEPES, 20 mM KCl, and 30 mM MgCl₂. For determination of total adenine nucleotide content, 20 µl sample was incubated in 530 µl HEPES buffer, 48 µl 2 mM PEP (in HEPES buffer), and 2 µl 200 units/mg PK for 20 minutes at room temperature followed by inactivation at 80º C. For determination of ATP content, 20 µl sample was mixed with 580 µl HEPES buffer. Lumat LB 9507 (Berthold, Oak Ridge, TN)
luminometer was programmed to inject 100 µl working mix per sample. A
corruption was used to calculate the amount of ATP and ADP in the
samples.

Statistics

SigmaStat (Aspire Software International, Ashburn, VA) was used for all
statistical calculations. Means are expressed with standard error. The
independent two-sample t-test or one-way analysis of variance (ANOVA) was
used for continuous variables as appropriate. The $\chi^2$ test was used for
categorical variables. Statistical significance is at $p<0.05$. 
RESULTS

Demographics

The migraine and control groups are similar in some respects. The mean age of subjects in the migraine group was 42.9 ± 1.2 years and the mean age in the control group was 45.0 ± 1.6 years. There are no statistically significant differences in mean age (t-test, p=0.30), racial composition (one way ANOVA, p=0.74), and frequencies of diabetes ($\chi^2$ square, p=0.98), hyperlipidemia (p=0.92), hypertension (p=0.77), and stroke (p=0.41) between groups. There is, however, a significantly increased frequency of self-reported mood disorder in the migraine group (41.9% vs. 7.5%, p<0.001). Numbers of subjects with these conditions are shown in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Numbers of Subjects</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Migraine</td>
<td>Control</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Mood Disorder</td>
<td>28</td>
<td>5</td>
</tr>
<tr>
<td>Stroke</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Hemorrhage

The migraine group reports bleeding more frequently than the control group in some areas (see Table 3). Subjects with migraine experience significantly more gum bleeding (66.7% vs. 40.0%, p=0.015), ecchymosis (34.4% vs. 0%, p<0.001), and gross hematuria (18.0% vs. 2.5%, p=0.041). There are no significant
differences (p>0.05) in epistaxis, excessive dental treatment-associated bleeding, gastrointestinal bleeding, and excessive surgical bleeding.

### Table 3: Bleeding

<table>
<thead>
<tr>
<th></th>
<th>Numbers of Subjects</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gum Bleeding</td>
<td>40</td>
<td>17</td>
</tr>
<tr>
<td>Ecchymosis</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Gross Hematuria</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal Bleeding</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Excessive Dental Treatment Bleeding</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Excessive Surgical Bleeding</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

On average, the migraineurs have heavier menstrual periods (see Table 4). They have higher scores on a pictorial blood loss assessment chart than the control group (p=0.024). The mean score for the migraine group on the assessment chart is 286.8±61.5 points (range 26-1800) vs. 103.3±12.9 (range 32-262) points for the control group. Based on Janssen’s criterion for menorrhagia (Janssen et al. 1995), with a score of greater than 185 points on a visual assessment chart for menstrual blood loss, 40.5% of the migraine group and 15.3% of the control groups had menorrhagia. The difference in frequency of menorrhagia between groups reaches statistical significance (p=0.049). More migraineurs than controls also have had hysterectomies or endometrial ablation for excessive bleeding (27.9% vs. 7.5%, p=0.024).

Subjects with migraine are also more likely to have endometriosis (p=0.027). 19.7% of migraineurs report having endometriosis compared to 2.5% of controls.
Table 4: Menstrual Bleeding

<table>
<thead>
<tr>
<th></th>
<th>Numbers of Subjects</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Migraine</td>
<td>Control</td>
</tr>
<tr>
<td>Menorrhagia (score &gt;185)</td>
<td>15 (N=36)</td>
<td>4 (N=26)</td>
</tr>
<tr>
<td>Hysterectomy or Endometrial Ablation</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

*Platelet Serotonin Content*

Platelet serotonin levels are measured in all subjects. The mean platelet serotonin content is 444.3(±42.5) ng per 10\(^9\) platelets for all migraine subjects and 560.9(±50.1) ng per 10\(^9\) platelets for all control subjects (Table 5). The absolute difference in the mean platelet serotonin content between groups becomes smaller when platelet serotonin in subjects taking a type of antidepressant medication called selective serotonin reuptake inhibitors (SSRIs) are excluded from calculations of means. When subjects taking SSRIs are not considered, the mean platelet serotonin content is 613.4(±46.8) ng per 10\(^9\) platelets for the migraine group and 655.7(±45.9) ng per 10\(^9\) platelets for the control group. Platelet serotonin levels are significantly lower in subjects using SSRIs compared to subjects that do not use SSRIs with p<0.001 for both control and migraine groups, but there are no statistically significant differences in mean platelet serotonin content between migraine and control groups regardless of SSRI use (p>0.05).
Table 5: Platelet Serotonin Content

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SSRIs (Yes/No)</th>
<th>Mean Platelet Serotonin (ng/$10^9$ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migraine</td>
<td>All*</td>
<td>444.3(±42.5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>144.7(±24.1)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>613.4(±46.8)</td>
</tr>
<tr>
<td>Migraine without Aura</td>
<td>All*</td>
<td>451.6(±58.9)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>140.6(±27.6)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>633.0(±68.5)</td>
</tr>
<tr>
<td>Migraine with Aura</td>
<td>All*</td>
<td>452.0(±57.4)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>173.6(±49.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>581.9(±54.5)</td>
</tr>
<tr>
<td>Control</td>
<td>All*</td>
<td>560.9(±50.1)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>127.6(±24.4)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>655.7(±45.9)</td>
</tr>
</tbody>
</table>

*All includes subjects who are taking SSRIs and subjects who are not taking SSRIs.

Dense Granule Enumeration

Platelets in migraineurs have significantly fewer dense granules than the control subjects (p<0.001), which is shown in Figure 3. On average, the migraine group has 1.61±0.14 dense granules per platelet while the control group has 3.85±0.14 dense granules per platelet.
Figure 3. **Mean Numbers of Dense Granules per Platelet in Migraine and Control Groups.** There is significant difference in the mean number of dense granules between migraine and control groups.

![Bar chart showing mean numbers of dense granules per platelet.](chart)

Table 6: Dense Granules

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SSRI (Yes/No)</th>
<th>Mean Number of Dense Granules per Platelet</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migraine</td>
<td>All*</td>
<td>1.61(±0.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.42(±0.21)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1.72(±0.18)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>All*</td>
<td>3.85(±0.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4.14(±0.41)</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3.78(±0.14)</td>
<td></td>
</tr>
</tbody>
</table>

*All includes subjects who are taking SSRIs and subjects who are not taking SSRIs.

Since the mean numbers of dense granules between subjects using SSRIs and those who do not use SSRIs do not differ in both migraine and control groups, the medications do not affect dense granule counts (Table 6). The number of
dense granules in platelets does not correlate with the serotonin content (R=0.104; p=0.312), as shown in Figure 4.

Figure 4. Correlation between Numbers of Dense Granules and Serotonin Stores.

Adenine Nucleotides

Table 7: Platelet ATP Content and ATP:ADP Ratio

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SSRI (Yes/No)</th>
<th>Mean Platelet ATP (µmole/10^{11} platelets)</th>
<th>Mean Platelet ATP:ADP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migraine (with and without aura)</td>
<td>All*</td>
<td>4.47(±0.40)</td>
<td>1.78(±0.07)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4.01(±0.48)</td>
<td>1.86(±0.09)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>4.73(±0.57)</td>
<td>1.73(±0.09)</td>
</tr>
<tr>
<td>Migraine with aura</td>
<td>All*</td>
<td>3.79(±0.41)</td>
<td>1.69(±0.11)</td>
</tr>
<tr>
<td>Migraine without aura</td>
<td>All*</td>
<td>4.88(±0.59)</td>
<td>1.83(±0.08)</td>
</tr>
<tr>
<td>Control</td>
<td>All*</td>
<td>3.94(±0.35)</td>
<td>1.73(±0.07)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.25(±0.30)</td>
<td>1.76(±0.17)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>4.10(±0.41)</td>
<td>1.73(±0.08)</td>
</tr>
</tbody>
</table>

*All includes subjects who are taking SSRIs and subjects who are not taking SSRIs.
By ANOVA, there is no difference in platelet ATP content (p=0.628) and ATP:ADP ratio (p=0.918) among the groups of subjects listed in Table 7.
One finding in this study is an increased bleeding tendency in women with migraine headache compared with control subjects that do not suffer from migraine headache. In this study, migraineurs had significantly higher incidence of menorrhagia, hematuria, gum bleeding, and ecchymoses than control subjects.

The study also shows decreased numbers of platelet dense granules in women with migraine without aura and with aura. Deficiency of dense granules, called δ-storage pool deficiency (δ-SPD) is associated with bleeding tendencies and prolonged bleeding time in spite of normal numbers of platelets in the circulation. The diagnosis of δ-SPD, a reduced number of platelet dense granules below the normal range of 4 to 6 dense granules per platelet is necessary along with bleeding diathesis.

Although migraineurs had fewer numbers of platelet dense granules than controls, there was no difference in platelet serotonin stores between the groups. This finding does not lend support to the hypothesis that serotonin stores in the bloodstream are deficient in female migraineurs. Nonetheless, it is important to note that some researchers have reported lower in platelet serotonin content between migraineurs and control subjects in the past (Nakano et al. 1993). Perhaps the previous reports of decreased platelet serotonin content in migraineurs may have been related to use of certain medications. There are studies showing reductions in platelet serotonin stores after chronic treatment
with antidepressants affecting the serotonin transporter SERT (Javors et al. 2000), (Kotzailias et al. 2004). In this study, only those subjects, mostly in the migraine group, who have been taking medications that inhibit the serotonin transporter, the channel through which serotonin enters platelets and neurons, had significantly reduced serotonin stores in their platelets.

Similar to the results of platelet serotonin content, differences in platelet ATP and ADP content were not found between migraine and control groups. However, the use of medications blocking SERT had no effect on platelet ATP and ADP content. It is important to note that adenine nucleotides enter platelets through a transporter different than that for serotonin; adenine nucleotides enter through multidrug resistance protein 4 (MRP4), which is responsible for pumping cyclic nucleotides and nucleotide analogs (Jedlitschky et al. 2004). Thus, dense granule storage of adenine nucleotides is not linked with storage of serotonin.

In short, there was no difference in the amounts of serotonin and adenine nucleotides found in platelets between migraine and healthy control subjects although migraine subjects were found to have fewer numbers of dense granules in their platelets than control subjects. Since platelets from migraineurs had the same total amounts of stored serotonin and adenine nucleotides as those from controls, it may be presumed that dense granules in migraineurs store larger amounts of serotonin and adenine nucleotides. This points to future work in studying the quantal release of serotonin from individual platelets of migraine sufferers.
Although a difference was found in the number of platelet dense granules in migraine subjects compared to control subjects, the pathogenesis of the migraine headache is not necessarily related to platelets. There could be differences in serotonin receptors, specifically the 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} receptors, to which triptans bind or in proteins in secondary messenger systems. A limited number of papers address polymorphisms in serotonin receptor genes in migraineurs. For example, one paper found a relationship between a particular polymorphism and headache intensity (Marziniak et al. 2007). Another study identified a polymorphism in the gene for a G protein linked with the serotonin receptors that affect response to triptans (Schurks et al. 2007). Since gene polymorphisms may influence headache severity and response to triptans, gene polymorphisms may be related to response to endogenous serotonin. Studies of gene polymorphisms may help elucidate the role of endogenous serotonin in modulation of migraine symptoms.


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Migraine headache is a disabling chronic condition, especially in women of childbearing age. Its etiology is currently unknown. The efficacy of serotonin receptor (5-HT1B/1D) agonists, triptans, in relieving attacks of migraine headache suggests a role for serotonin in the course of the headache. Platelets contain the largest reserve of serotonin stored in the body and thus, platelets may play a role in the etiology of migraine attacks. The literature is equivocal about the role of the platelet in migraine. This study has identified evidence of a bleeding diathesis, including menorrhagia, gum bleeding, gross hematuria, and ecchymosis, in migraineurs. Migraineurs also have fewer platelet dense granules in their platelets than healthy control subjects (1.61±0.14 vs. 3.85±0.14), but there are no significant differences in platelet serotonin and adenine nucleotide content.