Investigating the role of the primary motor cortex in the StartReact effect using transcranial magnetic stimulation

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Abstract

It is well-established that the presentation of a startling acoustic stimulus (SAS) simultaneous with the go-signal in a simple reaction time (RT) task results in significant RT reductions, while leaving movement kinematics essentially unaltered. While this phenomenon, termed the StartReact effect, has been extensively studied, cortical involvement in the neural mechanism underlying the RT-facilitation effects of a SAS remains widely debated. Applying sub-threshold TMS to motor areas results in increased cortical excitability and reductions in control RT. When this technique was used in a startle paradigm no RT benefits were seen, providing evidence that the cortex may not be involved in the StartReact effect; however, these results may also have been due to a floor effect of startle RT. It has been shown that RT in response to a SAS is significantly slower for complex movements, providing a possible method of distinguishing between these hypotheses. As such, the purpose of the experiments in this thesis was to determine if the application of sub-threshold TMS following a SAS when preparing to react with a complex movement would facilitate startle RT. If so, it would provide evidence for cortical involvement in the RT-facilitation effects of startle. The first experiment revealed that the task employed did not lead to an increase in RT in startle conditions, limiting the ability to make conclusions regarding the StartReact effect. In the second experiment the timing complexity of the task was increased, with the goal of increasing startle RT; however, startle RT was again not significantly slower for the complex movement than the simple movement. Furthermore, there was again no effect of TMS stimulation condition on startle RT. These results suggest that either the cortex does not play a role in the StartReact effect, or a floor effect of RT was reached in startle conditions; thus, alternative methods of investigating the neural mechanism underlying the RT-facilitation effects of startle are warranted.

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Glossary of Terms

ECR: Extensor carpi radialis

EMG: Electromyography

HSD: Honestly significant difference

HSP: Hereditary spastic paraplegia

ICF: Intracortical facilitation

ICI: Intracortical inhibition

IRI: Inter-response interval

M1: Primary motor cortex

MEP: Motor evoked potential

Ooc: Orbicularis orsi muscle

PA: Posterior-anterior

PMRF: Pontomedullary reticular formation

RM ANOVA: Repeated measures analysis of variance

rMT: Resting motor threshold

RT: Reaction time

SAS: Startling acoustic stimulus

SCM: Sternocleidomastoid muscle

TMS: Transcranial magnetic stimulation

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Statement of Contribution of Collaborators

I, Victoria Smith, hereby declare that I am the sole author of this Master's thesis. The conception and design of these experiments was completed by myself, in collaboration with my thesis supervisor Dr. Anthony Carlsen, and with input from my thesis committee consisting of Dr. Erin Cressman and Dr. Diane Ste-Marie. Data collection, data analysis and statistical analyses were completed by myself under the guidance of Dr. Anthony Carlsen, who also provided editorial corrections.

Chapter I: Literature Review

1. Introduction

The everyday lives of humans are characterized by the movements we make that allow us to interact with our environment and achieve a variety of goals from getting to work to cooking dinner. Since movement is an integral part of our everyday lives, one of the aims of the field of motor control is to understand how movements are prepared and executed. As such, motor control research is not only valuable in understanding movement in healthy populations, but is also of great importance in advancing our knowledge of motor disorders such as Parkinson's disease. The results of these studies can then be used to inform clinicians about the etiology of these disorders and to guide clinical practice. One research tool used in motor control is a startling acoustic stimulus (SAS), which allows researchers to investigate the processes underlying movement preparation and initiation. Startle research has provided a wealth of information about movement preparation and the pathologies of different movement disorders (Carlsen, Maslovat, & Franks, 2012); however, the mechanisms underlying the effect of startle on movement execution are currently debated.

This lack of knowledge poses a problem because information from startle research is used to understand the preparation of movements ranging from ballistic wrist extension (Valls-Solé, Rothwell, Goulart, Cossu, & Munoz, 1999) to the control of posture and gait (Nonnekes, Carpenter, Inglis, Duysens, & Weerdesteyn, 2015). In addition, research using startle in patients with various parkinsonian syndromes has provided information on the specific locus of their movement deficits, resulting in the proposal of startle as a diagnostic tool for clinicians (Carlsen, Almeida, & Franks, 2013; Valldeoriola et al., 1998). Without a complete understanding of how the facilitatory effect of startle on reaction time (RT) works, the conclusions drawn from this

research may provide incomplete, or worse, inaccurate explanations about movement preparation; thus, conclusions about which neural substrates are causing movement deficits may be unfounded. These issues highlight the need for further research into the neural mechanism underlying startle-facilitated movements to inform researchers about how movement preparation occurs, and clinicians about the causes of the movement deficits in various clinical populations. As such, the present literature review will outline the currently available literature on the use of a SAS, the possible mechanisms responsible for its effects, and possible methods for examining the pathway underlying the StartReact effect.

2. Reaction time

The field of motor control is concerned with understanding how the central nervous system is organized to produce coordinated actions, as well as how sensory information from the environment and body is used in the control of movement (Schmidt & Lee, 2011). One approach to studying motor control is to use the human information processing model (for a review see Schmidt & Lee, 2011), which can be simplified into three separate stages based on the seminal work of Donders (1969). The first stage, *stimulus identification*, involves detecting and identifying that a stimulus has occurred. In the second stage, termed *response selection*, the individual decides what response to make based on the identified stimulus in the first stage. The final stage is called *response programming* and is when the individual prepares and then initiates the selected action. These information processing stages can occur within various parts of the central nervous system (e.g., cortex, brainstem, cerebellum) and involve the processing of knowledge of the surrounding environment provided by the senses about the movements and choices available to/required of an individual (i.e., information) (Cisek & Kalaska, 2010).

As information processing occurs inside the central nervous system, it cannot be directly observed; thus, Donders (1969) relied on reaction time (RT) experiments and behavioural results to elucidate the abovementioned processes. RT is calculated as the time from presentation of a stimulus to the beginning of a response; therefore, it has been suggested to represent the time needed to complete the stages of information processing (Donders, 1969). In his experiments, Donders relied on simple RT, go/no-go, and choice RT paradigms. In a simple RT task there is only one response option, whereas in a choice RT task there are at least two response options. In a go/no-go RT task, a response is required to one stimulus while no response is required to another stimulus (Donders, 1969). Due to differences in the information processing requirements following the go-signal in these different paradigms, Donders was able to provide some of the first evidence for these three stages of information processing, as well as their durations.

Since Donders' seminal experiments, the use of electromyography (EMG) in RT studies has allowed researchers to further partition RT into premotor and motor RT. Premotor RT refers to the time period between the presentation of the imperative stimulus and when muscle activity in the effector begins, whereas motor RT refers to the time interval between the onset of EMG activity in the movement effector and the beginning of the desired movement (Schmidt & Lee, 2011). Premotor RT is thought to better represent the central processes involved in generating a response, while motor RT can be affected by mechanical properties of the effector and other physiological processes occurring within muscles related to movement execution (Weiss, 1965). Various manipulations of the classic RT paradigms, such as the number of stimulus-response alternatives (Hick, 1952), stimulus-response compatibility (Fitts & Deininger, 1954), and movement complexity (Henry & Rogers, 1960), have provided researchers with further insight into the processes occurring when humans prepare and execute movements.

3. Movement complexity and response programming.

In their classic experiment, Henry and Rogers (1960) manipulated movement complexity by varying the number of movement components required in a simple RT paradigm. A movement component is defined as a movement, movements or an aspect of a movement that is programmed and processed as a single unit (Klapp & Jagacinski, 2011). Examples of a single component movement include a single button press or uttering a single word, whereas examples of a multiple component movement include a series of button presses or words. Henry & Rogers (1960) found that when the number of movement components increased, simple RT also increased. As their experimental protocol ensured that stimulus identification and response selection were the same between all levels of complexity, this suggests that the increase in simple RT was due to an increase in time spent programming the upcoming movement. Henry and Rogers captured this idea in their memory drum theory where they posited that after a movement has been programmed, the retrieval of more complex movements from memory takes longer and leads to increased simple RT.

Klapp et al. (1995, 2003; 1974) expanded on the work of Henry and Rogers in a series of experiments investigating the effects of movement complexity on simple and choice RT. Their results showed that when a response consisted of a single component movement (a single button press) the level of complexity did not affect simple RT. These results build on those of Henry and Rogers and provide evidence that information processing does not always proceed in a serial order. Specifically, these results provide evidence that in a simple RT paradigm participants are able to complete response programming prior to the go-signal, which has been termed pre-programming, or advance preparation (Klapp et al., 1974). The results of these studies also showed that with multiple component movements (four button press movements), simple RT

increased as movement complexity increased (Klapp, 2003), which suggests there is a limitation to what humans are capable of pre-programming.

Along this line, Maslovat et al. (2014) investigated the effects of timing complexity on simple RT by having participants complete three key-press movements where the duration of movement components was held constant, but either isochronous or non-isochronous timing was required between components. The isochronous movements had the same time interval between the end of each movement element and the beginning of the next, whereas the non-isochronous movements had a shorter time interval between the first and second movement elements than between the second and third movement elements. Non-isochronous movements, which require a more complex timing structure, had longer simple RT than isochronous movements, suggesting that timing preparation cannot be pre-programmed and presumably occurs during the RT interval in a simple RT paradigm. Maslovat et al. (2016) provided further support for this notion by employing the same three key-press movements in a study time paradigm, where participants were shown a pre-cue indicating the required movement and were allowed to choose how long the pre-cue was displayed until presentation of the imperative stimulus. The time between presentation of the pre-cue and the self-selected beginning of the trial is referred to as the study time, and is believed to reflect the amount of time required for advance response preparation (Immink & Wright, 2001). Results showed that increasing timing complexity did not increase study time, but did lead to an increase in simple RT, suggesting that rather than being prepared in advance, preparation of timing structure occurs during the RT interval.

The reason for the apparent inability to pre-program movement timing is not fully understood; however, two primary hypotheses have been proposed. The first hypothesis proposed is that a representation of timing must be immediately implemented and cannot be held

in working memory; thus, it must be programmed during the RT interval, resulting in slower RT for movements with greater timing complexity (Maslovat et al., 2016; Maslovat, Klapp, et al., 2014). In contrast, the second proposal suggests that increases in RT due to increased timing complexity may not be due to preparation processing occurring during the RT interval, but rather due to increased time required for initiation processing (Maslovat et al., 2016; Maslovat, Klapp, et al., 2014). Specifically, in a simple RT paradigm, preparatory neural activation is increased during the foreperiod to a level below movement threshold. Following the presentation of the imperative stimulus, initiation processes provide additional neural activation, resulting in movement execution (Hanes & Schall, 1996). Movements with greater timing complexity may result in: 1) a lower level of neural activation being maintained during the foreperiod, 2) a lower rate of initiation-related activation accumulation or 3) a combination of the two (Kennefick, Maslovat, Chua, & Carlsen, 2016)**.** Any of these situations would increase the amount of time required for initiation processes to occur, resulting in slower RTs for movements with more complex timing structure. Kennefick et al. (2016) used transcranial magnetic stimulation (TMS), which can elicit motor evoked potentials (MEPs) that can be used as an index of corticospinal excitability, to investigate excitability of the motor pathway during the RT interval in simple and complex movements in a simple RT paradigm. Results showed that corticospinal excitability increased at a slower rate, and began to increase later in the RT interval for complex movements than for simple movements. As the complex movement required greater timing complexity, these results suggest that timing complexity effects on simple RT may be due to timing preparation occurring during the RT interval, as well as initiation processes beginning later and occurring at a slower rate. While the exact mechanism remains unclear, these results indicate that researchers

using a simple RT paradigm can use different levels of timing complexity in their experimental task to elicit relatively faster or slower simple RTs depending on their research question.

4. The StartReact effect

Another well-established method for studying advance preparation in a simple RT paradigm involves the use of a SAS (for a discussion of the method see Carlsen, Maslovat, Lam, Chua, & Franks, 2011). When a SAS is presented concurrent with the go-signal in a simple RT task it leads to significant reductions in RT, while still producing the intended movement (Valls-Solé et al., 1999). This phenomenon has been termed the StartReact effect, and it is believed to arise due to the involuntary triggering and release of a prepared movement (Carlsen et al., 2012; Valls-Solé, Kumru, & Kofler, 2008). This effect has been seen in a variety of tasks including, but not limited to, ballistic wrist extension (Carlsen, Chua, Inglis, Sanderson, & Franks, 2004a), anticipatory postural adjustments (MacKinnon et al., 2007), saccades (Castellote, Kumru, Queralt, & Valls-Solé, 2007) and sit-to-stand movements (Queralt, Valls-Solé, & Castellote, 2008). Based on this, the use of a SAS has become an invaluable tool for researchers investigating which aspects of a movement can be pre-programmed, in which situations pre-programming can occur, and how movement preparation is affected in various clinical populations (for a review see Carlsen et al., 2012).

4.1. The startle response. When studying StartReact it is important to ensure that participants are in fact being startled by the SAS, otherwise it cannot be said that the effect of a startle is being studied. The startle reflex is a protective response elicited by an intense, unexpected stimulus that is characterized by a stereotypical pattern of whole body muscle flexion activity (Landis, Hunt, & Strauss, 1939). When a SAS is presented, the sensory input is sent from the cochlear nucleus to the pontomedullary reticular formation (PMRF), which generates

descending activity within startle-related spinal motor neurons via the reticulospinal tract and results in the reflexive startle response (Yeomans & Frankland, 1995). This pathway bypasses cortical processing in the auditory cortex that usually accompanies auditory stimuli, resulting in the rapid onset of the startle response (14-151 ms) (Yeomans & Frankland, 1995). The startle response involves activity within numerous muscles and although many of these can be used as a startle indicator, the eye blink response measured in the orbicularis oculi (Ooc) muscle and/or activation in the sternocleidomastoid (SCM) muscle are commonly used by researchers (Brown et al., 1991). Despite being frequently used as a startle indicator, it has been suggested that activation in the Ooc may not be a reliable indicator of a startle response (Carlsen, Dakin, Chua, & Franks, 2007). For example, Carlsen et al. (2007) showed that the EMG configuration of the eyeblink was significantly different depending on whether or not an SCM response was also elicited. In addition, even when the rest of the startle response has habituated, the presentation of a SAS will elicit activity in Ooc, indicating that participants are exhibiting the auditory eyeblink response rather than an auditory startle eyeblink, which is thought to involve a different circuit/pathway (Brown et al., 1991). These results highlight methodological issues about the validity of using the Ooc as a startle indicator in experiments investigating the StartReact effect. The SCM is among the first detectable parts of the startle response following the eyeblink, is typically the last aspect of the startle response to habituate to repeated presentation of a startle, and is the most reliable startle indicator other than an eyeblink (Brown et al., 1991). Furthermore, it has been shown that when the RT results of trials with SCM activity (SCM+) and those without SCM activity (SCM-) are considered separately, the results are drastically different (Maslovat, Franks, Leguerrier, & Carlsen, 2015). As such, experiments examining or utilizing

the StartReact effect should use EMG activity in the SCM muscle as their indicator of whether or not participants were startled (c.f. Marinovic, de Rugy, Lipp, & Tresilian, 2013).

4.2. Support for the StartReact effect. Before a SAS could be used to study advance programming, it was necessary to ensure that it really was leading to reductions in RT due to the *involuntary release* of the *intended* and *prepared* movement:

4.2.1. Involuntary release. Since many studies using a SAS present it in conjunction with the go-signal, the possibility that it was simply speeding up the voluntary release of the movement rather than leading to the involuntary release of a movement was put forth. Regardless of whether the SAS was presented 150, 500, or 1500 ms prior to any go-signal being presented in a simple RT task, the prepared movement was elicited approximately 90 ms following the SAS with no significant differences in RT between the three time points (Carlsen & MacKinnon, 2010; MacKinnon, Allen, Shiratori, & Rogers, 2013). The ability of a SAS to lead to the release of the intended movement when presented during the foreperiod, independently of the go-signal, indicates that a SAS leads to the involuntary release of prepared movements.

4.2.2. Intended response at early RT. Another proposed explanation for the StartReact effect was that a SAS leads to an initial startle response at the early RT latency and the voluntary movement is then superimposed onto this response at the normal latency of voluntary movements (Valls-Solé et al., 1995). In order to investigate this possibility, Valls-Sole et al. (1999) compared the EMG burst timing patterns of control and startle movements and found there were no significant differences between the conditions in terms of EMG burst durations or inter-burst intervals (see also Carlsen, Chua, Inglis, Sanderson, & Franks, 2004b). The lack of

EMG differences indicates that StartReact RTs are not due to superimposition of a voluntary response onto an initial startle response.

4.2.3. Prepared movement. To determine if the presentation of a SAS leads to a movement that has been prepared in advance, Carlsen et al. (2004a) compared the effects of presenting a SAS in a simple versus choice RT paradigm. In accordance with previous results, a SAS presented in a simple RT task led to significantly reduced RT; however, there was no RT facilitation in the choice RT task. The differing results between simple and choice RT provide evidence that pre-programming is required for a SAS to lead to significant reductions in RT. Further support that a SAS leads to the release of a pre-programmed movement comes from an investigation of the effect of a SAS on multiple different movements. When participants were required to perform either a 20^o, 40^o or 60^o wrist extension movement on a given trial in a simple RT paradigm, the presentation of a SAS always led to the early and rapid release of the correct amplitude movement (Carlsen et al., 2004b). Taken together, these studies indicate that a SAS leads to the release of a movement that has been pre-programmed in advance of the go-signal.

5. The mechanism underlying the StartReact effect

Despite the abundance of knowledge that has been obtained about the StartReact effect, the neural mechanism underlying this phenomenon is still widely debated. Multiple different hypotheses have been put forth; however, as of yet there is no definitive evidence available to explain the RT-shortening effects of a SAS. The four primary hypotheses that have been forwarded are intersensory facilitation, stimulus intensity, subcortical storage, and cortical storage. Each of these hypotheses will be briefly discussed.

5.1. Intersensory facilitation. It was originally suggested that the StartReact effect may not be related to startle and was instead an example of the well-known phenomenon of

intersensory facilitation (Valls-Solé et al., 1995). Intersensory facilitation refers to the significant RT shortening that occurs when an imperative stimulus is accompanied by an accessory stimulus of another modality presented concurrently or at approximately the same time (Nickerson, 1973). While there is no direct evidence to refute this claim, the RT reductions associated with intersensory facilitation are in the range of 20 to 50 ms (Nickerson, 1973), which are substantially less than the ≥70 ms RT reductions seen with a SAS (Valls-Solé et al., 1995). In addition, the StartReact effect is often seen in experiments where an auditory imperative stimulus is used, so it is not possible for any intersensory facilitation to occur. As the StartReact effect cannot be fully explained via intersensory facilitation, it is unlikely this is the primary mechanism.

5.2. Stimulus intensity. It was also hypothesized that the StartReact effect may be an exaggerated case of a stimulus intensity effect (Carlsen et al., 2004a). This was put forth as a possible mechanism as it is well established that as the intensity of an imperative stimulus increases, the latency of the response to that stimulus is reduced (Woodworth, 1938). As a SAS generally consists of a sound with an intensity level of 120-130 dB, it seems plausible that the intensity is high enough that it results in RTs as fast as those seen in startle experiments. Carlsen et al. (2007) tested this hypothesis by varying the intensity of the go-signal in a simple RT paradigm from 83 to 123 dB in 10 dB steps, and comparing the RT trials where a startle response was observed in SCM (SCM+) and trials where no startle response was seen (SCM-). In SCM- trials, RT decreased as stimulus intensity increased, with RTs reaching ~100 ms in the 113 dB and 123 dB conditions. In contrast, RT in SCM+ trials decreased to ~80 ms regardless of the stimulus intensity, indicating a dissociation between startle and StartReact responses. These findings provide strong evidence against the hypothesis that the StartReact effect is an

extraordinary case of the stimulus intensity effect; rather it is a separate phenomenon specifically related to effects of a startle.

5.3. The sub-cortical storage hypothesis. With speeded sensory effects ruled out as the primary mechanism underlying startle facilitation of RT, two main hypotheses emerged to explain how faster response execution may underlie the StartReact effect (see Appendix A for an illustration). The first hypothesis suggested that the presentation of a SAS may directly trigger the release of a sub-cortically stored motor program (Carlsen et al., 2004a; Valls-Solé et al., 1999). Releasing a motor program from sub-cortical structures would allow cortical processing associated with voluntary movements to be bypassed, resulting in faster RTs. It has been shown in numerous studies that RTs in response to a SAS can be well below 100 ms (Carlsen et al., 2009; Carlsen, Lam, Maslovat, & Chua, 2011; Maslovat, Carter, Kennefick, & Carlsen, 2014; Valls-Solé et al., 1999). Valls-Sole et al. (1999) stated that due to these extremely short RTs (as fast as 65 ms) there was not enough time for the involvement of the cortical pathway in these startle-elicited responses. This was based on the fact that 35 ms are required for an auditory stimulus to reach the auditory cortex and 20 ms of efferent conduction time are required for a motor command to reach the forearms, leaving only 10 ms for processing of the movement. The PMRF was suggested as a possible site for sub-cortical motor program storage as it is known to be part of the pathway for both the startle reflex and voluntary movement (Rothwell, MacKinnon, & Valls-Solé, 2002). It has also been shown that the fastest startle RTs occur when there is also a burst in the SCM, which is part of the startle response and is known to originate from sub-cortical structures including the PMRF (Carlsen et al., 2007). Both of these studies provide compelling evidence that prepared movements can be stored sub-cortically; however, neither of these studies can give direct evidence of the involvement of sub-cortical structures.

Investigation of the effects of a SAS in clinical populations has provided further support for the sub-cortical storage hypothesis. Honeycutt & Perrault (2012) investigated the StartReact effect in a group of individuals who had suffered a stroke that damaged areas of their cortex and corticospinal tract. On control trials, the stroke patients exhibited delayed response latency and abnormal muscle activation, as seen by inappropriate flexor activity resulting in uncoordinated muscle patterns. In contrast, when a SAS was presented there were no differences in RT, muscle activation pattern, or elbow position between the stroke patients and healthy individuals. As these patients have damage to their corticospinal tract and cortex, these results point to the idea that the StartReact effect leads to the release of a sub-cortically stored motor program without any involvement of the cortex (Honeycutt & Perreault, 2012). Yet, it is important to note that the patients in these studies suffered from chronic stroke and it is known that the central nervous system is capable of adapting over time in response to injury (Stein & Hoffman, 2003); thus, their neural pathways may not accurately represent those seen in healthy individuals.

The StartReact effect has also been studied in individuals suffering from hereditary spastic paraplegia (HSP), which is a disease characterized by retrograde degeneration of axons within the corticospinal tract, especially in the projections to the lower limbs (Nonnekes et al., 2014). Nonnekes et al. (2014) found that on control trials HSP patients had significantly longer ankle dorsiflexion RTs than healthy participants, but the presentation of a SAS led to similar RTs between healthy individuals and those with HSP. These results provide further evidence for the release of a sub-cortically stored motor program, as individuals with HSP have degraded corticospinal fibers and intact reticulospinal tracts, but exhibit normal responses to a SAS. When considering these results, it is important to remember that the patients may have remaining corticospinal fibers, and results in patient populations may not accurately represent how the brain

normally controls movement. Despite the limitations of the aforementioned studies, taken together their results provide reasonable evidence for the sub-cortical hypothesis.

5.4. The cortical storage hypothesis. In contrast to the sub-cortical storage hypothesis, recent research has provided evidence that the cortex may in fact play an important role in the StartReact effect, which has led to the generation of a second competing hypothesis for the StartReact mechanism. Alibiglou and MacKinnon (2012) applied supra-threshold TMS to the contralateral primary motor cortex (M1) during the RT interval following a SAS. When high-intensity TMS is applied during an isometric contraction it leads to an MEP approximately 20 ms after the TMS pulse, followed by suppression of EMG activity for 100-300 ms, termed the silent period. The first 50 ms of this silent period is caused by reduced spinal motor neuron excitability, while suppression after 50 ms is caused by a decrease in cortical excitability and an interruption of voluntary drive within the cortex (Fuhr, Agostino, & Hallett, 1991). A similar reduction in corticospinal activity occurs 50-200 ms following supra-threshold TMS applied at rest (Tergau et al., 1999)**.** As such, if supra-threshold TMS is applied more than 50 ms before agonist EMG burst onset in a RT paradigm, any increases in RT can be attributed to disruption of voluntary cortical drive. Alibiglou & Mackinnon (2012) applied supra-threshold TMS 70 ms prior to each participant's mean RT as measured during a block of control trials (i.e., non-startle and startle trials with no TMS). If a SAS leads to the release of a motor program sub-cortically, without the involvement of the cortex, high-intensity TMS should not have any influence on startle RT. However, their results showed a significant delay of both control and startle RT when TMS was applied, while the onset of SCM activity in response to the SAS, which is known to be sub-cortical, was not affected by TMS, suggesting that M1 is somehow involved in the StartReact effect. To further support their findings, Alibiglou and MacKinnon (2012) measured Hoffman

reflexes and found no suppression of spinal excitability following TMS, indicating that inhibition of spinal motor neurons was not responsible for their RT results. These results were the first to provide evidence that the mechanism underlying the early response-triggering effects of a SAS may include the cortex.

As previously mentioned, application of supra-threshold TMS also leads to an MEP, which can be used as an index of corticospinal excitability (Rossini et al., 2015). Loud sounds, such as a SAS, usually lead to motor cortical suppression and a reduction in corticospinal excitability, as evidenced by reductions in MEP amplitude (Furubayashi et al., 2000; Ilic et al., 2011). Based on this, Marinovic et al. (2014) applied TMS following presentation of a SAS in an anticipation timing task expecting that MEP amplitude would be reduced, which would provide evidence that startle-facilitation of RT is due to the release of a sub-cortically stored motor program. In contrast, the presentation of a SAS led to increased corticospinal excitability, suggesting that the cortex is involved in the neural pathway underlying the StartReact effect. This study also showed that spinal facilitation, as measured using transcranial electric stimulation, cannot account for these findings, further supporting a role of the cortex in startle-facilitation of RT (Marinovic et al., 2014). A limitation of studies using supra-threshold TMS is that the effects of TMS can extend to the reticular formation (Fisher, Zaaimi, & Baker, 2012), allowing for the possibility that the observed results are due to sub-cortical rather than cortical inhibition.

Additional support for the involvement of the cortex in the StartReact effect is provided by Stevenson et al. (2014). The authors used a vocalization task that required participants to produce a prepared syllable in a simple RT paradigm with a SAS being presented on random trials. Speech preparation involves both cortical and sub-cortical brain regions, including the

motor cortex (Price, 2010)**,** and speech initiation/execution processes have been shown to be driven by cortical projections onto sub-cortical areas via a cortico-subcortical descending pathway (Simonyan & Horwitz, 2011). Results showed that a SAS led to significantly reduced RT for the vocalization task. Due to the highly cortical nature of vocalization tasks, these results support a role of the cortex in the StartReact effect. When considering these results, it is important to remember that despite vocalization relying heavily on the cortex, this does not provide direct evidence that the cortex is involved in the StartReact effect pathway. Despite the aforementioned limitations of these studies, their results provide evidence that the contribution of cortical structures to the StartReact effect cannot be ignored, and it is likely that both sub-cortical and cortical structures are involved in the startle pathway.

These findings led to the proposal that a SAS leads to an involuntary increase in initiation-related activation processes via an ascending sub-cortical pathway, triggering the release of the motor program that has been prepared and stored within the cortex without engaging in the usual cortical processing (Carlsen et al., 2012) (see Appendix A for illustration). This proposal is based on a model where preparation and initiation are separate processes, with preparation involving increasing activation to a level below movement threshold and initiation involving the input of additional activation to surpass this threshold (Hanes & Schall, 1996). Within this model, a motor program is conceptualized as a neuronal network with strengthened synaptic connections, termed a cell assembly, and preparation involves activating these neurons to a level below threshold (Wickens, Hyland, & Anson, 1994). Considering the StartReact effect within this model, participants engage in preparation processes during the foreperiod that lead to activation of the desired cell assembly. Presentation of the SAS then provides the additional activation needed to reach threshold and lead to movement execution. The reason for the

exceptionally fast RTs seen is that the SAS directly activates initiation processes via an ascending reticulo-thalamo-cortical pathway, leading to activation of the cell assembly without engaging in the usual cortical processing (Carlsen et al., 2012).

Indirect support for this sub-cortically mediated pathway comes from research investigating the relative contributions of voluntary and startle initiation-related activation to the StartReact effect. Maslovat et al. (2014) presented a SAS at various times during the RT interval and found that the pattern of RT results were consistent with an additive-activation model where control and startle-related activation are summed before response generation. These results point to the existence of a common structure underlying initiation processes for both voluntary movement and involuntary startle processes. The authors suggest that these additive processes likely occur within a cortical structure and lead to release of movements via the corticospinal tract (Maslovat, Carter, et al., 2014). This initial experiment utilized an auditory go-signal, but replication of the protocol with a visual go-signal yielded a similar pattern of RT results that can be explained via an additive-activation model (Maslovat, Drummond, Carter, & Carlsen, 2015). In combination with initial results implicating the role of cortical structures in the pathway of the StartReact effect, these results provide evidence for the hypothesis that the neural mechanism of SAS-facilitated RTs may be due to sub-cortical triggering of a cortically stored motor program. The presence of evidence for both the sub-cortical and cortical storage hypotheses, combined with the lack of indirect evidence for either, has left the mechanism underlying the StartReact effect elusive, warranting an alternative method of investigating this phenomenon.

6. Transcranial magnetic stimulation (TMS)

TMS is a non-invasive neurostimulation technique that allows researchers to transiently alter neural activity in order to investigate processes occurring within the cortex (Rossini et al.,

2015). TMS was first introduced by Barker at al. (1985) as an alternative to the neurostimulation technique of transcranial electric stimulation, and has since become a widely used tool in both research and clinical settings. TMS has gained popularity because it uses the principle of electromagnetic induction to create an electric current in neural tissue in a simpler and less painful manner than transcranial electric stimulation (Barker et al., 1985). A TMS machine consists of coils of copper wire connected to a capacitor that can discharge by the click of a switch, leading to a large current flowing through the coil for approximately one millisecond. This current creates a rapidly changing magnetic field such that when the coil is placed over the head, the magnetic field is able to penetrate the scalp and skull, inducing an electric current within the cortex that activates nearby neurons (Rothwell, Thompson, Day, Boyd, & Marsden, 1991). Depending on the type of TMS and the stimulation parameters used, the application of a magnetic field can have many different effects on processes within the cortex. If a single TMS pulse is of a high enough intensity, it can depolarize neurons and lead to action potentials. Alternatively, when pulses are applied repeatedly at various frequencies it can alter resting cortical excitability and can thus be used as a therapeutic tool (Rossi, Hallett, Rossini, & Pascual-Leone, 2009). This has allowed researchers to investigate a variety of cortical processes to determine how the brain functions in both healthy and clinical populations (Rossini et al., 2015).

6.1. Single pulse TMS. Single pulse TMS consists of the application of a single magnetic stimulation pulse over the brain. Application of single pulse TMS over M1 can lead to descending neural activity in the corticospinal tract, which can be measured through epidural spinal recordings (Nakamura, Kitagawa, Kawaguchi, & Tsuji, 1997). At relatively high intensities (180-200% of active motor threshold), TMS leads to the generation of an initial spinal volley called the D-wave (Di Lazzaro et al., 1998). This D-wave is created by direct activation of

the axons of pyramidal tract neurons of the corticospinal tract (Patton & Amassian, 1954). At lower intensities (up to 150% of active motor threshold), secondary volleys termed I-waves are induced by indirect activation of pyramidal tract neurons via monosynaptic connections with cortical neurons (Patton & Amassian, 1954). The descending activity that is generated depends on the intensity of the TMS, as well as the direction of the induced electric current (Di Lazzaro et al., 2012). When studying motor processes, TMS is most commonly applied to generate a posterior-anterior current, which leads to a single descending I-wave at low intensities. As intensity increases, later I-waves are seen, and at very high intensity an initial D-wave is seen (Di Lazzaro, Ziemann, & Lemon, 2008).

After propagating the corticospinal tract and spinal cord to the motor neurons, the effects of TMS can lead to muscular activity termed an MEP. MEPs provide a method of measuring the level of excitability within the cortex, as evidenced by increases in MEP amplitude during motor preparation and reduced amplitude in individuals with damage to their motor cortex due to stroke or other diseases (Rossini et al., 2015). In order to elicit an MEP, TMS must, by definition, be applied at a supra-threshold intensity. Supra-threshold TMS is any intensity greater than an individual's motor threshold, which is the minimum intensity required to generate an MEP (Rossini et al., 1994). In contrast, a TMS pulse that is applied at a sub-threshold intensity will not result in the generation of an MEP, or any descending corticospinal activity (Di Lazzaro & Rothwell, 2014), although it may still result in measureable effects on MEPs (see section 6.3 – 6.4). These different methods and effects of TMS have provided a wealth of information about how humans prepare for upcoming movements, where and when information processing occurs within the cortex, and how the brain adapts to be able to control movement following injury, among many other uses (Rossini et al., 2015).

6.2. Safety. Before any form of TMS is employed in an experiment there are a few important safety issues that must be considered. One possible severe side effect resulting from TMS is seizures; however, their occurrence is very rare (Rossi et al., 2009). Reported seizures occurred in response to repetitive TMS applied at supra-threshold intensities, and in individuals taking medication known to increase the risk of seizures (Rossi et al., 2009). In contrast, the present experiments utilized single-pulse TMS that was primarily of a sub-threshold intensity. In addition, when TMS is applied in accordance with published safety guidelines it is considered very safe. As such, participants should complete a safety screening questionnaire prior to receiving TMS. This questionnaire eliminates participants who are at increased risk for seizures (e.g., people with epilepsy), people on medication that may increase their risk of a seizures, people with metal in their head, and women who are pregnant, among others (Rossi, Hallett, Rossini, & Pascual-Leone, 2011). In studies where the TMS coil will be held on a participant's head for a longer period of time, it is possible that participants will get a mild headache; however, this is generally due to the pressure of the TMS coil against the scalp rather than the effects of TMS on the cortex (Rossi et al., 2011). As such, if participants pass the screening questionnaire and are permitted breaks within the experiment, TMS can be administered in a completely safe and painless matter.

6.3. Paired-pulse TMS. Paired-pulse protocols, where two TMS pulses are applied through the same coil to the same location of the brain in short succession, have been used to investigate cortical excitability and intracortical circuits. Intracortical inhibition (ICI) involves the application of a sub-threshold conditioning stimulus 1-6 ms prior to a test stimulus and results in significant reduction in MEP amplitude (Kujirai et al., 1993). This phenomenon occurs due to activation of a low-threshold inhibitory cortical circuit by the conditioning pulse, which

inhibits the generation of action potentials created by the supra-threshold test pulse (Ilić et al., 2002). Using a similar protocol, but with application of the conditioning stimulus 6-30 ms before a supra-threshold test pulse results in significantly larger MEPs elicited by the test pulse in the targeted muscle, termed intracortical facilitation (ICF) (Chen, 2004; Kujirai et al., 1993; Ni & Chen, 2011). Comparisons of these two protocols have shown that they have different conditioning stimulus thresholds, different responses to changes in coil orientation, and are affected differently by drugs, supporting the notion that they are two separate phenomena (Ziemann, Rothwell, & Ridding, 1996). With respect to ICF, it has been shown that an inter-stimulus interval of 9-15 ms leads to the greatest amount of facilitation in most individuals (Du, Summerfelt, Chiappelli, Holcomb, & Elliot Hong, 2014). These results were obtained with a conditioning stimulus intensity of 80% resting motor threshold. As the intensity of the conditioning stimulus increases above 80%, facilitation becomes greater; however, the likelihood of the conditioning stimulus eliciting an MEP also increases as the intensity increases (Ziemann, Lonnecker, Steinhoff, & Paulus, 1996; Ziemann, Rothwell, & Ridding, 1996). As such, researchers investigating ICF generally employ a conditioning stimulus of 80-90% of resting motor threshold applied 10 to 15 ms prior to a testing stimulus (Kossev, Siggelkow, Dengler, & Rollnik, 2003; Rossini et al., 2015).

The facilitation seen in an ICF paradigm may be due to enhancement at a cortical, sub-cortical, or spinal level as interactions at each of these levels contributes to the generation of MEPs. As its name suggests, ICF is believed to occur as a result of activation of a low-threshold facilitatory cortical circuit for a brief period of time following the conditioning TMS pulse (Kujirai et al., 1993). Conditioning stimuli that lead to clear MEP facilitation have no effect on H reflexes, which indicates that the enhancing effects are not due to processes occurring at a spinal

level (Di Lazzaro et al., 2006; Ziemann, Rothwell, et al., 1996). Similarly, when an anodal electric test stimulus is used a magnetic conditioning stimulus (TMS pulse) does not lead to facilitation (Chen et al., 1998). As anodal electric stimulation directly affects corticospinal neurons (Day et al., 1989), these results suggest that the magnetic conditioning pulse is not acting through sub-cortical structures, rather the mechanism resulting in ICF when magnetic pulses are used is due to a cortical mechanism**.** Use of imaging technology has given researchers another method of investigating where ICF effects originate. Strafella & Paulus (2001) used positron emission topography to show that the classic ICF paradigm results in an increase in regional cerebral blood flow in M1, which provides further (albeit indirect) evidence that it is a cortical mechanism. Finally, direct recordings of descending volleys in the cervical spinal cord following ICF have provided information about its exact effects on corticospinal activity. As previously mentioned, TMS that induces a posterior-anterior current direction preferentially evokes I-waves, which reflect the excitability of the motor cortex (Nakamura, Kitagawa, Kawaguchi, & Tsuji, 1996). An ICF paradigm leads to facilitation of the later I-waves of the test pulse, but does not affect the D-wave, which further suggests that ICF is a cortical mechanism (Nakamura et al., 1997). In response to the conditioning stimulus presented alone there are no discernable descending volleys in the spinal cord, which has led to the suggestion that the facilitation of test pulses is due to disperse descending activity from the cortex (Di Lazzaro et al., 2012; Di Lazzaro & Rothwell, 2014). Although the mechanism underlying ICF is not fully understood, these abovementioned findings provide strong evidence that ICF is due to interactions occurring within cortex.

6.4. Sub-threshold TMS and reaction time. The effects of TMS on RT have been extensively studied, and it has been found that these effects vary depending on the intensity of

TMS used, as well as the time of TMS application. When sub-threshold TMS is applied to the contralateral M1 early in the RT interval (0 – 60 ms following the go-signal) it leads to significant RT facilitation (Pascual-Leone, Brasil-Neto, Valls-Sole, Cohen, & Hallett, 1992; Pascual-Leone, Valls-Sole, et al., 1992; Sawaki, Okita, Fujiwara, & Mizuno, 1999; Soto, Valls-Sole, & Kumru, 2010). These reductions in RT have been argued to be caused by two possible processes; the first is the previously mentioned intersensory facilitation effect (Terao et al., 1997). In addition to passing a stimulating current to the cortex there is an auditory click and a vibration of the scalp associated with TMS, both of which can act as an additional cue to initiate a movement, resulting in faster RT. A second way sub-threshold TMS may speed up RT is by adding extra activation to M1, which facilitates the transfer of a motor program to the neurons responsible for task execution (Pascual-Leone, Valls-Sole, et al., 1992). Experiments including sham TMS conditions (providing the sensory aspects of TMS without any added magnetic energy) have provided evidence that the RT shortening effect of sub-threshold TMS may simply be due to intersensory facilitation. For example, TMS applied to the ipsilateral M1 or parietal cortex resulted in similar reductions in RT as TMS applied to the contralateral M1 (Burle, Bonnet, Vidal, Possamai, & Hasbroucq, 2002; Sawaki et al., 1999). Yet, an experiment by Soto, Valls-Sole and Kumru (2010) provides compelling evidence that sub-threshold TMS reduces RT by affecting the cortical processes involved in RT. Participants performed both simple and choice RT tasks with sub-threshold TMS being applied during the RT interval, with TMS having a similar shortening effect on RT in both tasks. Investigation of the locus of intersensory facilitation and automatic alerting effects on RT has shown that these effects are caused by speeding up processing of the perceptual aspects of RT (Hackley & Valle-Inclan, 1998). As the motor processes are the same in choice and simple RT paradigms, while the perceptual processes

are not, the similar results for the simple and choice RT tasks of Soto et al. (2010) suggest that TMS affected motoric processes rather than perceptual processes. These results provide support for the notion that TMS-induced reduction of RT is largely due to added energy facilitating motor processing in M1.

While these experiments provide conflicting results about the mechanism underlying the RT shortening effects of sub-threshold TMS, more recent research suggests that it is due to an additive effect of these processes. In a series of experiments, Smith and Carlsen (2017; see Appendix B) applied sub-threshold TMS or sham TMS over M1 early in the RT interval (30 ms following the go-signal) of a targeted wrist extension movement. Results showed that both real and sham TMS significantly reduced RT; however, real TMS also resulted in significantly reduced RT in comparison to sham TMS. These effects were seen when a click of the same sound intensity and profile was used as the sham condition, as well as when a TMS pulse was applied to the same location as real TMS, but with a latero-medial current direction (as opposed to the posterior-anterior current direction used in the real TMS condition). As the second sham condition fully replicated all aspects of the sensory experience of TMS, these results provide evidence that while intersensory facilitation plays a role in RT speeding, there is also a direct effect of sub-threshold TMS on the cortex.

Despite several studies suggesting that there is a direct effect of sub-threshold TMS on the cortex that results in RT speeding, the mechanism underlying this effect is not fully understood. Experiments investigating the effect of sub-threshold TMS applied following the go-signal on RT have used intensities of 90% resting motor threshold (Nikouline, Ruohonen, & Ilmoniemi, 1999; Sawaki et al., 1999) or the highest intensity that does not elicit an MEP (Hashimoto, Inaba, Matsumura, & Naito, 2004). These intensities are similar to the intensity of

the conditioning stimulus used to elicit MEP facilitation in the ICF paradigm (Kossev et al., 2003; Kujirai et al., 1993). Considering the hypothesis that sub-threshold TMS adds energy to M1 within the ICF paradigm, sub-threshold TMS applied during the RT interval may add extra energy to the motor pathway resulting in RT facilitation in the same manner it leads to MEP facilitation. Specifically, this added energy would raise the level of activation of preparation processes closer to threshold resulting in less time required for initiation processes to reach this threshold, and faster RTs. Smith and Carlsen (2017; see Appendix B) investigated this hypothesis by measuring the degree of ICF elicited in participants and compared the RT pattern in response to sub-threshold TMS between individuals who displayed significant ICF and those who did not. Results showed a similar RT pattern in both of these groups, suggesting that the facilitatory effects of sub-threshold TMS on RT and MEPs do not share a common underlying mechanism.

As previously mentioned, Pascual-Leone et al. (1992) originally proposed that the facilitatory effect of sub-threshold TMS on RT was due to the added energy in M1 facilitating the transfer of the stored motor program to the neurons responsible for movement execution. Analysis of the EMG traces obtained when sub-threshold TMS was applied at varying times following the go-signal revealed no significant differences between TMS conditions, despite significant reductions in RT (Hashimoto et al., 2004). The authors suggested that the added energy provided by TMS results in facilitation of temporal processing within M1 without affecting the cortically stored motor program, providing support for the hypothesis of Pascual-Leone et al. (1992). Thus, it appears that sub-threshold TMS applied early in the RT interval results in RT-shortening due to a combination of intersensory facilitation and a direct facilitatory effect of TMS on temporal processing within M1.

7. Summary

While there is extensive research trying to elucidate the mechanism underlying the StartReact effect, the presence of evidence for both the sub-cortical and cortical storage hypotheses, combined with the lack of direct evidence for either, has left the mechanism underlying the StartReact effect elusive. These mixed results have led to a debate over the mechanism underlying this RT speeding effect of startle (Appendix A) and this represents a significant gap in the motor control field that needs to be addressed. Without a full understanding of the mechanism underlying this phenomenon it is difficult to appropriately interpret what startle results mean with respect to human movement preparation. Research in non-human primates has shown that supra-threshold TMS results in descending activity in both the reticulospinal and corticospinal tracts (Fisher et al., 2012), which has led proponents of the sub-cortical storage hypothesis to dismiss the results of studies in support of the cortical storage hypothesis (Nonnekes et al., 2015). In response to this criticism, sub-threshold TMS, which affects the cortex but does not lead to any descending corticospinal activity, may be a viable way to determine if the cortex contributes to the StartReact effect.

8. Research question

The general objective of this thesis was to elucidate the mechanism underlying the observed RT shortening effect of a SAS. Specifically, I investigated whether or not sub-threshold TMS affects RTs associated with startle triggered movements. If sub-threshold TMS does facilitate startle RT, it would provide evidence for cortical involvement in the pathway of startle-triggered movements. This paradigm was previously used by Smith & Carlsen (2017; see Appendix B), who had participants perform a ballistic wrist extension in response to an auditory go-signal in a simple RT paradigm, and on 25% of trials a SAS was presented simultaneously

with the go-signal. On a subset of trials sub-threshold or sham TMS was applied over M1 30 ms following the go-signal in control trials, and at -15, 0, +15 or +30 ms with respect to the SAS in startle trials. As noted above, in control trials sham TMS lead to significantly faster RT than trials with no TMS; however, real TMS also lead to significantly faster RT compared to the sham condition, supporting the notion that TMS affects cortical processes. Notably, in startle trials, there were no RT differences between startle alone, startle with sham TMS, or startle with real TMS. Two interpretations can be made from these findings: 1) the cortex has limited involvement in the StartReact effect or 2) the RT for a SAS-triggered wrist extension movement is the fastest speed at which humans are capable of responding, as the RTs in all startle conditions were approximately the same across all experiments. Based on this second interpretation, I used movements with more complex timing requirements, which are known to result in increases in both control RT (Klapp, 2003) and startle RT (Maslovat, Klapp, et al., 2014). It was expected that this procedure would provide a larger available range (~30 ms) for RT facilitation by the sub-threshold TMS in the startle conditions in order to determine if cortex does indeed contribute to RT in these conditions. As such, the purpose of this experiment was to examine if the application of sub-threshold TMS to M1 early in the RT interval following a SAS for a complex movement would lead to further facilitation of startle RT. It was hypothesized that when a more complex movement was used, sub-threshold TMS would lead to facilitation of startle RT, providing evidence that the cortex (specifically M1) is involved in the mechanism underlying the StartReact effect (see Appendix C).

Chapter II: Research Article

Sub-threshold transcranial magnetic stimulation does not facilitate reaction time in startle conditions, regardless of movement complexity

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Abstract

The presentation of a startling acoustic stimulus (SAS) in conjunction with the go-signal in a simple reaction time (RT) task results in the involuntary triggering of the prepared movement at a significantly reduced latency. While this phenomenon, termed the StartReact effect, has been well-documented, cortical involvement in the neural pathway underlying the RT-speeding effect of a SAS remains widely debated. Previous research has shown that sub-threshold transcranial magnetic stimulation (TMS) applied during the RT interval results in significant RT reductions in control trials, whereas TMS had no effect on startle RT. These results suggest that the cortex has limited involvement in the StartReact effect; however, it is also possible that the use of a simple wrist movement resulted in a floor effect on RT. To test this, a complex movement, which has been shown to result in increased RT in startle trials, was used to provide a greater available range for facilitation by TMS. As such, the purpose of the present experiment was to determine if sub-threshold TMS applied early in the RT interval would result in facilitation of startle RT during a complex task. In two separate experiments, participants completed three-key press movements of varying difficulty in a simple RT paradigm in response to an auditory go-signal, which was randomly replaced by a SAS on 25% of trials. On a subset of trials TMS or sham TMS was applied over the motor cortex 30ms following the go-signal and 15ms following the SAS in control and startle trials, respectively. Results showed that in control trials both real and sham TMS facilitated RT; however, there was no effect of either stimulation condition on startle RT. These results suggest that either the cortex has limited involvement in the neural mechanism underlying the StartReact effect, or a floor effect of RT was reached.

1. Introduction

In a simple reaction time (RT) paradigm, the presentation of an acoustic stimulus that is loud enough (typically >120 dB) to evoke a classical startle reflex (Brown et al., 1991) simultaneous with the imperative go-signal results in the involuntary, early triggering of a prepared movement (Carlsen, Maslovat, & Franks, 2012; Valls-Solé, Kumru, & Kofler, 2008). These RT reductions are achieved while maintaining similar movement kinematics as in control trials, and this phenomenon has been termed the StartReact effect (Carlsen, Chua, Inglis, Sanderson, & Franks, 2004b; Valls-Solé, Rothwell, Goulart, Cossu, & Munoz, 1999). This phenomenon has been seen in a wide variety of movements ranging from ballistic wrist extension (Carlsen, Chua, Inglis, Sanderson, & Franks, 2004a) to sit-to-stand movements (Queralt, Valls-Sole, & Castellote, 2008). A startling acoustic stimulus (SAS) has also been used to study movement preparation and initiation processes in both healthy (MacKinnon et al., 2007; Oude Nijhuis et al., 2007; Siegmund, Inglis, & Sanderson, 2001) and motor disordered populations (Carlsen, Almeida, & Franks, 2013; Honeycutt & Perreault, 2012; Nonnekes et al., 2014). Despite the abundance of research obtained through the use of the startle paradigm, the neural mechanism underlying the StartReact effect remains widely debated (Carlsen et al., 2012; Marinovic & Tresilian, 2016).

It was originally proposed that a SAS led to the involuntary triggering of a prepared motor program that was stored within sub-cortical structures, bypassing the cortical pathway employed in voluntary movements (Carlsen et al., 2004a; Valls-Solé et al., 1999). This was based on the finding that RTs in response to a SAS could be as fast 65 ms, while the sensorimotor conduction time for wrist responses is approximately 55 ms, which does not leave sufficient time for the involvement of a transcortical pathway (Valls-Solé et al., 1999). However,

research employing transcranial magnetic stimulation (TMS) has provided evidence that contradicts this hypothesis (Alibiglou & MacKinnon, 2012; Stevenson et al., 2014). Application of high-intensity TMS over the contralateral primary motor cortex (M1) interrupts voluntary cortical drive and reduces cortical excitability for a brief period of time, a phenomenon termed the cortical silent period (Fuhr, Agostino, & Hallett, 1991). Alibiglou & Mackinnon (2012) applied high-intensity TMS over M1 in a simple RT paradigm, and their results showed that TMS not only slowed down control RT, it also led to significantly slower RT in startle trials. As TMS interrupts cortical processing, these results suggest that the cortex must be involved in the neural pathway underlying the facilitatory effects of startle on RT. This led to the proposal that a SAS results in an involuntary increase in initiation-related activation processes via an ascending reticulo-thalamo-cortical pathway, triggering the release of a cortically stored motor program without engaging in usual cortical processing (Carlsen et al., 2012).

Despite evidence that the cortex may be involved in the RT speeding effects of startle, it has been shown that high-intensity TMS leads to descending drive in both the cortico-spinal and cortico-reticular pathways (Fisher, Zaaimi, & Baker, 2012). This has led to the suggestion that the TMS-induced increases in startle RT may have been due to reduced reticulospinal excitability rather than reduced corticospinal excitability, providing further evidence for the sub-cortical storage hypothesis (Nonnekes, Carpenter, Inglis, Duysens, & Weerdesteyn, 2015). Using lower intensity (sub-threshold) TMS is a possible alternative method for investigating cortical involvement in the StartReact effect. Application of a sub-threshold TMS pulse 6-30 ms prior to a supra-threshold test pulse leads to significant facilitation of the motor evoked potential (MEP), termed intracortical facilitation (ICF) (Chen, 2004; Kujirai et al., 1993; Ni & Chen, 2011).

In contrast to high intensity TMS, *sub-threshold* TMS does not lead to any discernable descending volleys in the spinal cord (Di Lazzaro & Rothwell, 2014). Furthermore, when sub-threshold TMS is applied over M1 early in the RT interval, it leads to significant facilitation of RT (Burle, Bonnet, Vidal, Possamai, & Hasbroucq, 2002; Pascual-Leone et al., 1992; Soto, Valls-Sole, & Kumru, 2010). It has been argued that this RT speeding effect of sub-threshold TMS may be due to either a direct effect of adding extra energy to M1 (Pascual-Leone et al., 1992), or simply due to intersensory facilitation (Nickerson, 1973), as TMS pulses result in an auditory click and vibration of the scalp. However, it was recently shown in a series of experiments that intersensory facilitation can only explain part of the facilitatory effect of sub-threshold TMS on RT; there is an additional direct effect of TMS on the cortex (Smith & Carlsen, 2017; see Appendix B). When sub-threshold TMS or sham TMS, consisting of a TMS pulse of the same intensity applied over vertex or M1 with an altered current direction, were applied 30 ms following the go-signal RT was significantly faster than in the no TMS condition, but real TMS was also faster than the sham TMS condition. These results provide evidence that the facilitatory effect of TMS on RT is due to an additive effect of intersensory facilitation, and a direct effect of TMS on the cortex. Despite this direct effect of TMS seen in control trials, there was no effect of TMS on startle RT, regardless of the time of TMS application with respect to the SAS. These results appear to suggest that the cortex is not involved in the StartReact effect, providing evidence for the sub-cortical storage hypothesis; however, it is also possible that the startle RTs for a targeted wrist extension movement represent the fastest speed at which humans are capable of responding. This alternative interpretation is supported by the finding that startle RT was approximately 80 ms across all experimental conditions in all three experiments (Smith & Carlsen, 2017; see Appendix B),which precludes the ability to definitively state that the cortex

was not involved in the StartReact effect based on these results. It has previously been shown that the use of a multiple component movement in a simple RT paradigm results in significant RT increases in both control (Klapp, 2003) and startle conditions (Maslovat, Klapp, Jagacinski, & Franks, 2014), providing a possible method of distinguishing if the aforementioned results were caused by a lack of cortical involvement in the StartReact effect, or if a floor effect was reached. Increasing startle RT would provide a greater available range for facilitation by sub-threshold TMS, allowing an investigation of cortical involvement in the StartReact effect without the limitations encountered by previous studies employing a simple task, or supra-threshold TMS.

Based on this, the purpose of the present experiment was to determine if the application of sub-threshold TMS over M1 early in the RT interval in a simple RT paradigm for a complex movement would result in facilitation of startle RT. In a first experiment, participants were required to perform a three-key press movement with the same time interval between the key presses. It was hypothesized that this movement would result in increased RT, and that sub-threshold TMS would result in reductions of startle RT. In a second experiment, participants were required to perform a three-key press movement with differing timing intervals between the key presses, as well as a single-key press movement. It was hypothesized that this movement would result in further increases in RT in comparison to the simple movement, allowing for greater reductions in RT due to application of TMS in both control and startle trials, and an investigation of cortical involvement in the StartReact effect.

2. Experiment 1 Methods

2.1. Participants. Eighteen adults with normal or corrected to normal vision and no obvious upper body abnormalities participated in this study. Prior to beginning the experiment,

all participants completed a TMS safety questionnaire and only those whose answers indicated they had no contraindications to TMS were allowed to participate (Rossi, Hallett, Rossini, & Pascual-Leone, 2011). The data from eleven participants was excluded from the analysis as these participants did not exhibit significant ICF. Furthermore, one participant did not exhibit consistent SCM activation in response to a SAS, resulting in a final sample size of six participants (3 male, 3 female; M_{age} = 27 years, SD = 9) being included in the final data analysis (see below for inclusion criteria details). All participants provided informed consent before participating, and the experiment was conducted in accordance with the ethical guidelines set by the Health Sciences and Science Research Ethics Board at the University of Ottawa, and conformed to the latest version of the Declaration of Helsinki.

2.2. Apparatus and task. Participants were seated in a padded chair approximately 1.5 m from a 24 inch LCD computer screen with their right arm abducted approximately 30^o and flexed approximately 90^o at the elbow. Participants' right forearm rested parallel to the floor in a custom-made arm rest in a semi-prone position such that their palm faced inwards (Figure 1). Participants performed a three-key press movement against a telegraph key (Ameco AM-K4B) in a simple RT paradigm by reacting as quickly as possible to an auditory imperative stimulus. Participants began from a neutral wrist position with the back of the hand resting against the telegraph key, which was mounted on the manipulandum and oriented vertically such that the direction of switch closure was in the transverse plane about the longitudinal axis. The key press movement resembled pressing a Morse code button; however, participants made the movements by extending the right wrist rather than their fingers. This movement was chosen as a SAS has been shown to elicit a StartReact effect more consistently for wrist movements than for finger movements (Carlsen, Chua, Inglis, Sanderson, & Franks, 2009). In addition, flexion is part of the

generalized startle response (Brown et al., 1991); thus, extension movements elicited at short latency are a better indicator that the prepared movement was triggered involuntarily (i.e. the StartReact effect), rather than the startle reflex itself leading to an early response.

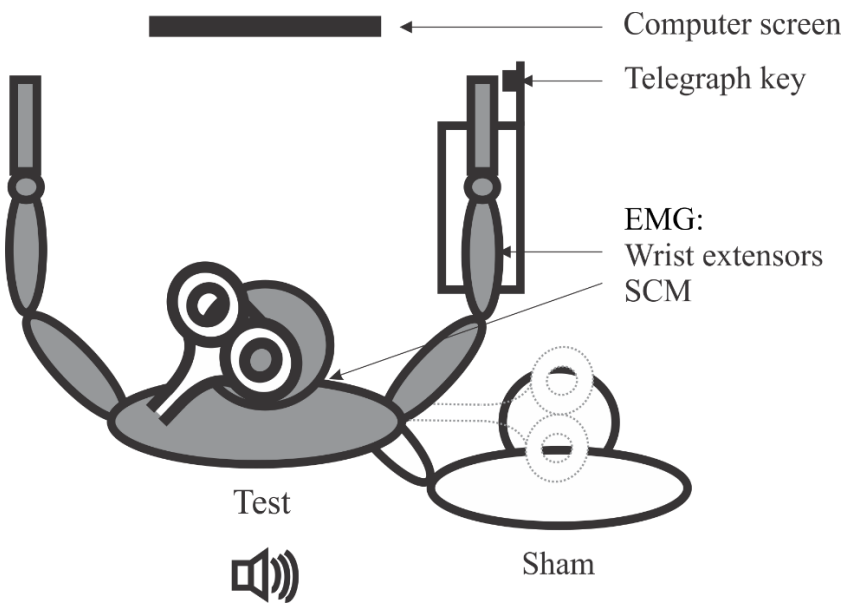

Figure 1. Participant set-up during experimental trials. Participants were seated with their arm in a custom-made manipulandum with the back of the hand resting against a telegraph key. In blocks with real TMS the coil was held to induce a posterior-anterior current direction over M1, and in blocks with sham TMS the coil was held to induce a latero-medial current direction over vertex.

The movement consisted of three key presses, each with a duration of 150 ms and an interval of 150 ms between each of the key presses - resulting in a total movement time of 750 ms. This movement was chosen because similar movements have been shown to consistently elicit a StartReact effect, but with slower control and startle RTs than simpler movements like a single ballistic wrist extension (Maslovat, Klapp, et al., 2014). The goal for each trial was to react as quickly and accurately as possible, and feedback regarding both RT and movement temporal accuracy was displayed on the monitor at the end of each trial. Participants were rewarded points for fast RTs (< 250 ms) and accurate movements, and were penalized for slow RTs (> 350 ms); however, points were only provided as an incentive and were not analyzed.

2.3. Transcranial magnetic stimulation. Single pulse TMS was delivered using a Magstim 200^2 stimulator with a figure-of-eight coil (D70mm, Magstim Company Ltd, Whitland, Dyefield, UK) to the contralateral M1 representation of the extensor carpi radialis (ECR) of the right forearm. To locate this area, the midpoint between the nasion and inion, and the left and right preauricular notches was found (this location was marked and used as the location of sham TMS application), followed by measuring four centimeters laterally and one centimeter anteriorly and marking this location. To find the optimal area for generating MEPs from the ECR, test pulses were delivered to locations near this mark in 0.5 cm steps to determine where the largest MEPs were found. The location that elicited the largest MEPs in the ECR was marked and saved by neuronavigation hardware and software (ANT Neuro Visor 2, Madison, WI). The resting motor threshold (rMT) for this area was then determined to the nearest 1% of stimulator output by finding the minimum intensity needed to elicit an MEP of 50 mV in five of ten trials (Rossini et al., 1994). All test pulses were delivered with the coil placed tangentially on the scalp and approximately perpendicular to the central sulcus, resulting in a posterior-anterior (PA)

current direction. During the paired-pulse MEP block prior to beginning the experiment TMS was delivered through two Magstim 200 stimulators connected in series via a Bistim module (Magstim Inc.). During the RT experiment single pulse TMS was delivered using an intensity of 85% of rMT, as this has been shown to lead to ICF while not producing an MEP (Kujirai et al., 1993). Sham TMS was also delivered at the same intensity of stimulator output in order to elicit the same sensory experience in both TMS and sham TMS conditions. In all blocks the neuronavigation system was used to allow the researcher to maintain the coil position within 1 mm of the marked ECR location.

2.4. Recording equipment. Surface EMG was collected from the superficial muscle bellies of the right ECR and right sternocleidomastoid (SCM) using bipolar preamplified (gain = 10) surface electrodes (Delsys Bagnoli DE-2.1) connected via shielded cabling to an external amplifier (Delsys Bagnoli-8). These electrodes were placed parallel to the muscle fibers and attached to the skin using double-sided adhesive tape. In addition, a grounding electrode (Dermatrode HE-R) was placed on the right lateral epicondyle. To minimize electrical impedance all electrode sites were cleaned with abrasive skin prepping gel and alcohol wipes prior to attachment. The telegraph key measured the duration of key presses and the time period between key presses through contact with the key, which required two N of force to close and showed a voltage of 10 V when open and 0 V when closed. Unfiltered EMG and telegraph key data were digitally sampled at 4000 Hz (National Instruments PCIe-6321) using a customized LabVIEW program and stored for offline analysis. Data collection was initiated by the computer for each trial 500 ms prior to presentation of the imperative stimulus and continued for 3000 ms.

2.5. Experimental procedure. Prior to beginning testing, 25 baseline MEPs were collected from the right ECR at an intensity equal to 120% of rMT for each participant. In addition, 25 MEPs were elicited using a conditioning stimulus intensity of 85% of rMT presented 12 ms prior to the test stimulus in order to assess ICF. The order of these MEP testing blocks was counterbalanced across participants. A customized LabView program was then used to calculate the peak-to-peak amplitude of MEPs for each participant, and these were compared between single pulse and paired pulse conditions using an independent samples Student's t-test; participants who did not exhibit ICF ($p > .2$) were excluded from participating in the remainder of the experiment. This was included as a screening measure to ensure that cortical excitability was being increased by the experimental parameters being used.

Participants who exhibited ICF then completed a block of 20 practice trials to allow them to become comfortable with the task. These practice trials were identical to experimental trials with the exception that there were no TMS or SAS trials. This was followed by the experimental trials, which consisted of eight blocks of 16 trials, for a total of 128 trials (see Figure 2A). Four of the eight blocks consisted of 10 control trials, two control trials with TMS, two SAS trials, and two SAS trials with TMS. The other four blocks consisted of 10 control trials, two control trials with sham TMS, two SAS trials, and two SAS trials with sham TMS. The blocks were broken up in this manner due to the sham TMS condition requiring movement of the TMS coil, which could not be done within a block as the experimenter was blind to the order of trials, and participants would have felt the coil being moved on their heads between trials. The order of blocks was randomly assigned and counterbalanced between participants. The number of trials was chosen to ensure participants were not exposed to the SAS too frequently (Carlsen, Maslovat, Lam, Chua, & Franks, 2011), but also to ensure there were enough TMS trials, which

increases the likelihood of obtaining a representative sample mean and minimizes within session variance (Orth, Snijders, & Rothwell, 2003).

Within each block, all trials began with the words “Get Ready!” being displayed on the computer screen for 1000 ms, followed by an auditory warning signal (100 ms, 200 Hz, 80 dB). After the warning signal the screen went blank and following a variable foreperiod of 1500 ms to 2500 ms the auditory imperative stimulus (82 dB, 25 ms, 1000 Hz sine wave) was presented (Figure 2B). All acoustic stimulus intensities were measured using an “A” weighted impulse setting of a sound level meter (Cirrus Research model CR:162C) at a distance of 30 cm from the loudspeaker. Both the warning signal and imperative stimulus were generated with digital to analog hardware (National Instruments PCIe-6321), and amplified and presented by a loudspeaker (MG Electronics M58-H, frequency response 300 Hz-11 Hz, rise time <1 ms) located 30 cm behind the participant, as measured on an individual basis from the opening of their auditory canal. After completion of the movement the participants’ feedback and their points earned/lost for the trial were displayed for 3500 ms until the beginning of the next trial. Feedback consisted of displacement RT (time from presentation of the imperative stimulus to the first key-press), and a graphical representation of their completed movement pattern displayed below the goal movement pattern. A customized LabVIEW (National Instruments Inc.) program controlled the timeline for each trial, as well as the display of information to the participant.

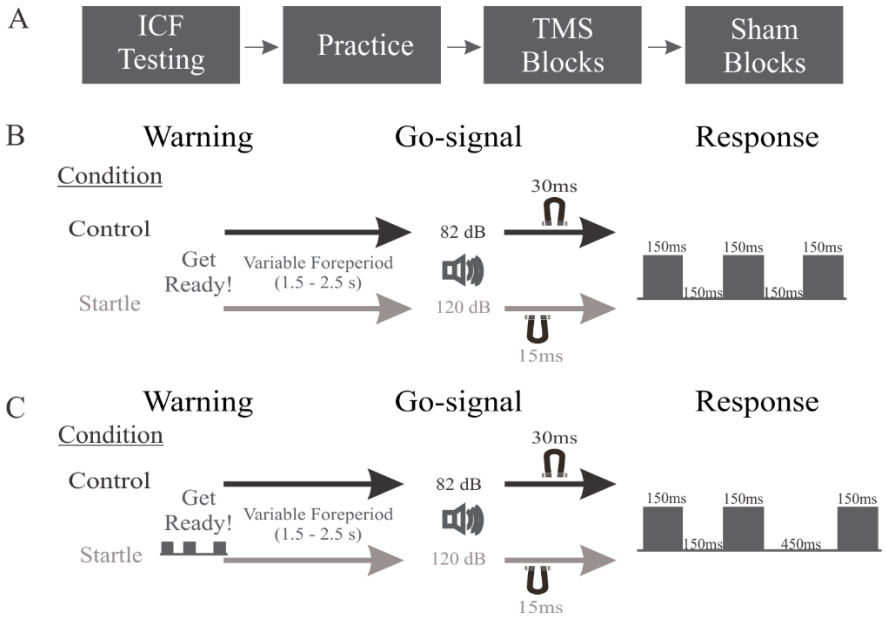

Figure 2. A) Timeline of the experimental session. Following the set-up of EMG and TMS, participants completed two ICF testing blocks, followed by a block of practice trials and eight blocks (four each of real and sham TMS) of the experimental trials. The order of real TMS and sham TMS blocks was counter-balanced across participants. *Note*: in Experiment 2 participants completed six experimental blocks (three each of real and sham TMS). B) Timeline of experimental trials in Experiment 1. An auditory warning signal and go-signal were separated by a 1.5 – 2.5 second variable foreperiod. The go-signal in control trials was 80 dB, and in startle trials this was replaced by a 120 dB startling acoustic stimulus. In a subset of trials TMS or sham TMS was applied 30 ms following the go-signal or 15 ms following the SAS in control and startle trials, respectively. C) Timeline of experimental trials for the complex movement in Experiment 2. Experiment 2 followed the same timeline as Experiment 1, with the exception that the to-be completed movement was displayed with the 'Get Ready' screen. For the simple movement, a single button press template was displayed on the 'Get Ready!' screen.

In control trials with TMS, the TMS pulse was applied 30 ms after presentation of the imperative stimulus with the current flowing in a PA direction. Control trials with sham TMS employed the same timing, but TMS was applied to elicit a latero-medial current direction over vertex. This was used as the sham condition because neuroimaging studies have shown that TMS over vertex does not interfere with task-related activity within M1 (Jung, Bungert, Bowtell, & Jackson, 2016), and the full sensory experience (ie. auditory click and vibration of the scalp) must be replicated in sham conditions to control for the non-specific effects of TMS (Duecker & Sack, 2015).

In startle trials, the control imperative stimulus was replaced by a SAS (120 dB, 25 ms, white noise waveform). SAS trials that included TMS or sham TMS were the same as control trials with the exception that TMS was applied 15 ms following the presentation of the SAS. These times were chosen for TMS application based on an accumulator model of neural activation, which indicates that initiation-related activation processes begin approximately 15 ms earlier in startle trials than in control trials (Maslovat, Carter, Kennefick, & Carlsen, 2014). The order of trials was controlled by a computer and was pseudorandomized such that a SAS was never presented on the first two trials of a block, and a SAS was never presented on two consecutive trials.

2.6. Data reduction and analysis. Startle trials where there was no discernible SCM activation were discarded, as this is considered to be a robust and reliable indicator that a startle response was elicited (Carlsen, 2015; Carlsen, Dakin, Chua, & Franks, 2007). SCM activation was defined as an SCM burst (see below for EMG burst onset detection criteria) occurring within 50-120 ms of presentation of a SAS (Carlsen et al., 2011). Once these trials were discarded, the percentage of startle trials where SCM activation was present was analyzed, and participants who

showed SCM activation on less than 50% of startle trials were excluded from data analysis. In addition, trials where participants were anticipating the go-signal or not paying sufficient attention to the task, indicated by RTs faster than 50 ms or slower than 350 ms, respectively, were discarded. Forty-five trials were discarded due to lack of SCM activation on SAS trials, 3 trials due to inattentiveness and 13 trials due to anticipation. This resulted in an overall inclusion rate of 91% (697/768).

The main dependent measure was premotor RT, measured as the time between presentation of the imperative stimulus and the time of EMG onset of the right ECR. Premotor RT was used as it is believed to represent central processing related to movement preparation following the go signal while excluding movement execution processes (Schmidt & Lee, 2011), and to allow for comparison with previous experiments investigating the effects of an SAS on RT. EMG burst onset for both the ECR and SCM was defined as the point where EMG activity reached two standard deviations above baseline level and remained elevated for at least 20 ms (Hodges & Bui, 1996). EMG offset was measured and defined as the point where EMG activity dropped below 20% of its maximal amplitude reached in that EMG burst. EMG traces were displayed on a computer monitor along with EMG onset and offset markers computed using a custom LabView algorithm and then manually adjusted to correct for any possible errors due to the strictness of the algorithm (i.e. marking a small muscle twitch prior to movement as EMG burst onset). In addition, peak EMG amplitude was defined as the greatest EMG amplitude that occurred within 100 ms of EMG burst onset.

Task performance was also examined to ensure participants were performing the desired movement in both control and startle trials. The inter-response interval (IRI) was measured as the time between the end of a key-press and the beginning of the following key-press. As such, the

time between the end of the first and the beginning of the second key press was defined as IRI-1 and the time between the end of the second and the beginning of the third key-press was defined as IRI-2. Key-press duration was also measured for each of the three key presses, and all of these variables were calculated from voltage changes recorded by the telegraph key.

Finally, MEP amplitude in the ICF testing blocks was defined as the greatest peak-to-peak amplitude recorded in a 30 ms window beginning 15 ms following the TMS pulse. MEPs were rejected if 1) root mean square EMG activity in the 50 ms preceding the TMS pulse exceeded twice the resting root mean square value for that trial (determined from a mean of the first 50 ms of EMG in the trial) or 2) if at any point prior to TMS application root mean square EMG exceeded 10 mV. MEPs were also rejected if peak-to-peak amplitude was less than 0.05 mV. This resulted in exclusion of 13 MEPs and an overall inclusion rate of 96% (287/300).

2.7. Statistical analysis. The proportion of SAS trials where an SCM response was elicited was analyzed using a one-way, three-factor (TMS: none, TMS, sham) repeated measures analysis of variance (RM ANOVA) to determine if there was a difference in startle incidence across conditions. Premotor RT, IRI-1, IRI-2, and key-press durations were analyzed using separate 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) RM ANOVAs to determine if there were any differences in RT or movement execution due to the presentation of a SAS, TMS or sham TMS. The effects of SAS, TMS and sham TMS on EMG burst duration and peak amplitude of the initial ECR burst were also analyzed using separate 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) RM ANOVAs. A Shapiro-Wilk test of normality indicated that MEP peak-to-peak amplitude was not normally distributed; thus, MEP amplitude was analyzed using a Wilcoxon Signed-Rank test to determine if there was significant MEP facilitation in the paired pulse TMS trials. The significance value

for all statistical tests was set at $p < .05$, and any significant differences were analyzed using a Tukey's Honestly Significant Difference (HSD) post-hoc test to determine the locus of the significant difference. Partial eta squared (η^2_p) values were calculated for all significant differences to determine the magnitude of the effects.

3. Experiment 2 Methods

3.1. Participants. Seven adults with normal or corrected to normal vision, no obvious upper body abnormalities and no contraindications to TMS (Rossi et al., 2011) participated in Experiment 2. One participant did not exhibit consistent SCM activation on startle trials, resulting in a final sample size of six participants (2 male, 4 female; $M_{age} = 24$, $SD = 2$). All participants provided written informed consent before participating in the experiment, which was conducted in accordance with the ethical guidelines of the Health Sciences and Science Research Ethics Board at the University of Ottawa, and conformed to the latest revision of the Declaration of Helsinki.

3.2. Experimental procedure. The experimental procedure was similar to that of Experiment 1 with three primary modifications: 1) an absence of exhibited ICF (determined during the ICF testing blocks prior to practice) was not used as an exclusion criterion, 2) the task required a more complex timing pattern, and 3) a single key press movement was included for comparison with the complex movement. While it was originally believed that the facilitation of both MEPs and RT by sub-threshold TMS may be due to a similar mechanism, our previous data showed no difference in RT facilitation patterns in response to TMS between individuals who exhibited ICF and those who did not (Smith & Carlsen, 2017; see Appendix B). As such, the ICF testing blocks were included to further test this hypothesis; however, participants who did not exhibit ICF were allowed to participate in the experiment to maximize the number of eligible

participants. Because the results of Experiment 1 revealed that the use of a three-key press movement with isochronous timing did not result in longer startle RTs than those seen in experiments employing a ballistic wrist extension movement, a task with a more complex timing requirement was employed in Experiment 2. Maslovat et al. (2014) found that movements with non-isochronous timing resulted in longer control and startle RTs in comparison to those with isochronous timing; thus, Experiment 2 used the same task with non-isochronous timing as used by Maslovat et al. In addition, a single key press movement was included in order to determine the magnitude of RT slowing resulting from the more complex movement as compared to the simple movement within the same group of participants.

Prior to beginning the experimental trials, two blocks of 25 MEPs were collected from the right wrist extensor. In one block of trials single pulse TMS was applied, while in the other block of trials paired-pulse TMS was applied (with the same TMS parameters as Experiment 1). Participants then completed 20 practice trials, 10 of each of the single and three-key press movements. The simple movement consisted of a single key press 150 ms in duration, whereas the complex movement consisted of three key presses of 150 ms duration, with a 150 ms interval between the first and second key presses, and a 450 ms interval between the second and third key presses, resulting in a total movement time of 1050 ms. The to-be-completed movement was displayed on the computer screen for 1000 ms prior to the beginning of each trial, accompanied by the words ‘Get Ready!’, indicating which movement to prepare. The remainder of each trial was identical to the trials in Experiment 1 (Figure 2B & 2C).

Following practice, the experimental trials were performed as six blocks of 24 trials, resulting in a total of 144 trials. Three of these blocks consisted of seven simple non-startle trials, two simple non-startle trials with TMS, seven complex non-startle trials, two complex non-startle

trials with TMS, one simple SAS trial, two simple SAS trials with TMS, one complex SAS trial and two complex SAS trials with TMS. The other three blocks consisted of seven simple non-startle trials, two simple non-startle trials with sham TMS, seven complex non-startle trials, two complex non-startle trials with sham TMS, one simple SAS trial, two simple SAS trials with sham TMS, one complex SAS trial and two complex SAS trials with sham TMS (Table 1). The order of blocks was counterbalanced across participants, and trials within blocks were presented pseudo-randomly, such that a SAS was never presented on the first two blocks of a trial, and a SAS was never presented on two consecutive trials. The rest of the experimental protocol (TMS, recording equipment and data reduction) was identical to that of Experiment 1. This resulted in the exclusion of 30 trials due to lack of SCM activation on SAS trials, 6 due to anticipation of the go-signal, 4 due to inattentiveness and 8 due to movement error, for a total inclusion rate of 95% (817/864). In addition, 49 MEPs from the ICF testing blocks performed prior to beginning the experiment were discarded, resulting in an inclusion rate of 84% (251/300).

Table 1. Summary of experimental trials in Experiment 2. Breakdown of total experimental trials by movement type, auditory stimulus and TMS stimulation condition completed by each participant.

		Movement Type/Trials	
Auditory Stimulus	TMS	Simple	Complex
Go (82 dB)	None	42 trials	42 trials
	Sham	6 trials	6 trials
	Real	6 trials	6 trials
SAS (120 dB)	None	6 trials	6 trials
	Sham	6 trials	6 trials
	Real	6 trials	6 trials

The statistical analyses performed in Experiment 2 were similar to those of Experiment 1, with some modifications due to the addition of a simple movement and the elimination of the ICF inclusion criteria. As the presence of ICF in the ICF testing blocks was not used as an inclusion criteria, independent Student's t-tests were performed to determine the proportion of participants who displayed significant ICF. Based on this, premotor RT was analyzed using a 3 (Subgroup: ICF, same, ICI) x 2 (Task: simple, complex) x 2 (Imperative stimulus: control, startle) x 3 (TMS: none, TMS, sham) mixed model ANOVA with repeated measures on task, imperative stimulus and TMS to determine if there was an effect of TMS or acoustic stimulus on RT, and if these effects differed between those who exhibited ICF and those who did not. The proportion of SAS trials where an SCM response was elicited was analyzed using a 2 (Task: simple, complex) x 3 (TMS: none, TMS, sham) RM ANOVA to determine if there was a difference in the incidence of startle responses elicited across conditions. To determine if there was any effect of imperative stimulus or TMS on movement accuracy or movement kinematics in the simple movement condition key press duration, initial ECR burst duration and initial ECR peak amplitude were analyzed using separate 2 (Imperative stimulus: control, startle) x 3 (TMS: none, TMS, sham) RM ANOVAs. The same analyses were completed for the complex movement condition, with each of the three key press durations being analyzed separately, as well as IRI-1 and IRI-2.

4. Experiment 1 Results

4.1. TMS. The mean resting motor threshold of all participants was 45% (SD = 8) of maximum stimulator output. This resulted in a mean TMS intensity of 38% (SD = 6) being used as the conditioning stimulus and a mean TMS intensity of 54% (SD = 8) being used as the test stimulus in the ICF testing blocks.

The mean peak-to-peak MEP amplitude from the ICF testing blocks can be seen in Figure 3. Analysis revealed that MEP amplitude was significantly greater for conditioned MEPs than unconditioned MEPs, $T = 0$, $p = .031$, $r = -.899$, indicating that, overall, participants exhibited significant ICF.

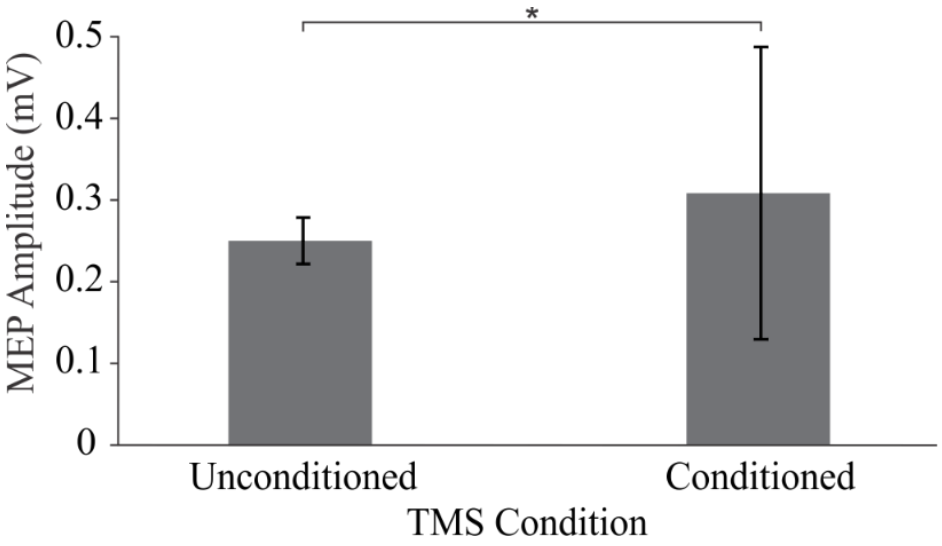

Figure 3. Mean (SE) motor evoked potential (MEP) peak-to-peak amplitude for control and conditioned TMS pulses in Experiment 1. Asterisks (*) denotes a significant difference between TMS conditions.

4.2. Premotor RT. Premotor RT values across all six experimental conditions can be seen in Figure 3. Analysis revealed a significant main effect of Imperative stimulus on RT, $F(1,5) = 41.207$, $p = .001$, $\eta^2_p = .892$, indicating that RT was significantly faster in startle trials than in control trials. There was no significant main effect of TMS ($p = .104$); however, there was a trend towards a significant interaction between the factors, $F(2,10) = 3.623$, $p = .066$, $\eta^2_p = .420$. This trend was likely due to both TMS and sham TMS resulting in shortened RT in the control condition (although not significantly), while there was no difference between TMS conditions in startle trials.

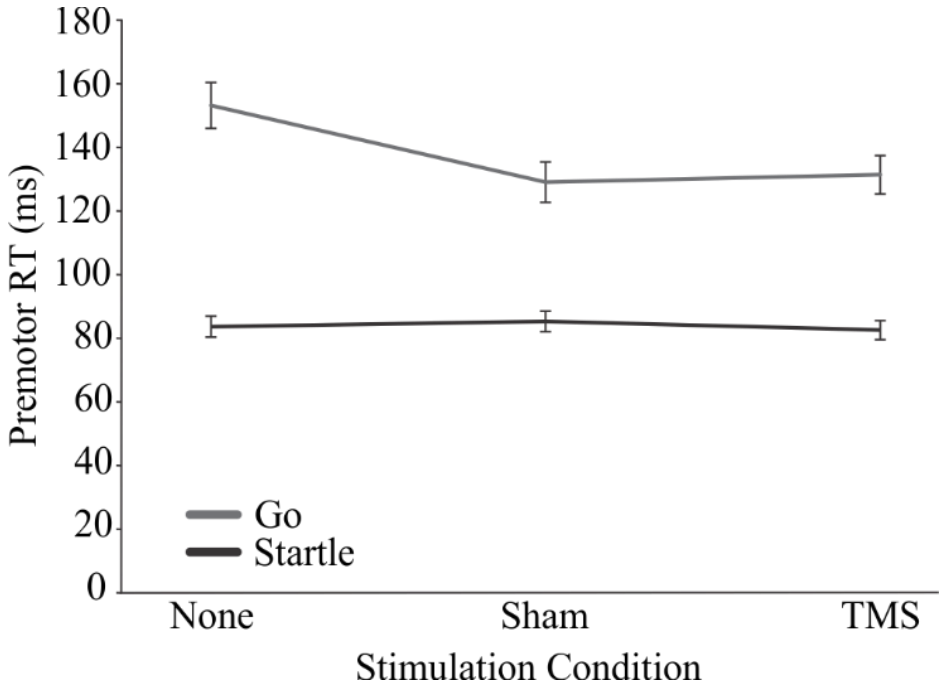

Figure 4. Mean (SE) premotor reaction time (RT) observed across TMS and acoustic stimulation conditions in Experiment 1. Premotor RT in the no stimulation (None), sham TMS, and real TMS conditions are shown as a function of acoustic stimulus condition (Control vs SAS).

4.3. Task performance. Analysis of the IRI-1 and IRI-2 revealed no significant differences between any of the experimental conditions ($p > .05$), indicating that participants performed similar interval timing across all conditions. Analysis of key press duration revealed a significant main effect of imperative stimulus on the duration of the first key press, $F(1,5) = 6.952$, $p = .046$, $\eta^2_p = .582$, indicating that the duration was significantly longer in the startle condition (132 ms, SD = 24) than in the control condition (112 ms, SD = 15). There was no significant effect of TMS and no interaction between the factors ($p > .05$). For key press two, there was a significant main effect of TMS, $F(2,10) = 8.378$, $p = .007$, $\eta^2_p = .626$; however, post-hoc analyses correcting for multiple comparison showed that there were no significant differences between TMS conditions ($p > .05$). There was also no significant effect of imperative stimulus and no interaction between the factors ($p > .05$). Finally, there were no significant differences between any of the experimental conditions for key press duration three ($p > .05$).

4.4. EMG activity. Analysis revealed a significant main effect of imperative stimulus on the duration of the initial ECR burst, $F(1,5) = 10.951$, $p = .021$, $\eta^2_p = .687$, revealing that burst duration was significantly shorter in the startle condition (83 ms, SD = 17) than in the control condition (97 ms, SD = 20). There was no main effect of TMS and no interaction between the factors ($p > .05$). Similarly, analysis of initial ECR peak amplitude revealed a significant main effect of imperative stimulus, $F(1,5) = 17.829$, $p = .008$, $\eta^2_p = .781$, indicating that peak amplitude was significantly greater in startle trials (0.148 mV, SD = 0.073) than in control trials (0.083 mV, SD = 0.042). There was also no significant effect of TMS or an interaction between the factors ($p > .05$).

4.5. SCM activity. The percentage of SAS trials where a startle response was elicited was 72% (SD = 20) in the startle without TMS condition, 90% (SD = 13) in the startle with TMS

condition, and 71% (SD = 19) in the startle with sham TMS condition. Analysis revealed no significant differences in the percentage of trials where a startle response was elicited ($p > .05$).

5. Experiment 2 Results

5.1. TMS. The mean resting motor threshold of all participants was 36% (SD = 6) of maximum stimulator output. This resulted in a mean TMS intensity of 30% (SD = 5) being used as the conditioning stimulus and a mean TMS intensity of 43% (SD = 8) being used as the test stimulus in the ICF testing blocks.

Analysis of the peak-to-peak MEP amplitude in the ICF testing blocks revealed that there was no significant difference between the unconditioned (0.421 mV, SD = 0.44) and the conditioned (0.535 mV, SD = 0.51) MEPs, $T = 9$, $z = -.314$, $p = .844$, $r = -.052$. However, Student's t-tests conducted within individuals revealed that two participants exhibited significant ICF, two participants showed no significant differences between conditioned and unconditioned MEP amplitudes and two participants exhibited significant intracortical inhibition (ICI) (unconditioned MEPs larger than conditioned MEPs). Based on this, subgroup (ICF, same, ICI) was included as a factor in the analysis of premotor RT.

5.2. Premotor RT. The premotor RT values for all twelve experimental conditions can be seen in Figure 5. Analysis revealed a significant main effect of imperative stimulus, $F(1,3) = 35.497$, $p = .009$, $\eta^2_p = .922$, as well as a significant main effect of TMS, $F(2, 6) = 28.724$, $p = .001$, $\eta^2_p = .905$. However, these main effects were superseded by a significant interaction between the factors, $F(2, 6) = 32.773$, $p = .001$, $\eta^2_p = .916$. Post-hoc analysis using Tukey's HSD indicated that in control trials RT was significantly slower in trials without TMS than in trials with either real or sham TMS ($p < .05$), with no significant difference between the two TMS conditions ($p > .05$). In contrast, there were no significant RT differences between any of the

TMS conditions in startle trials ($p > .05$). There was also a significant main effect of task, $F(1,3) = 14.169$, $p = .033$, $\eta^2_p = .825$, and a significant imperative stimulus x task interaction, $F(1, 3) = 15.91$, $p = .028$, $\eta^2_p = .841$. Post-hoc testing indicated that in control conditions premotor RT was significantly faster for the simple movement than the complex movement ($p < .05$), whereas there was no difference in RT between movements in startle conditions ($p > .05$). Finally, there were no significant differences or any interactions involving subgroup (all p-values > .05), indicating that the RT patterns did not differ depending on the presence of ICF or ICI in the ICF testing blocks.

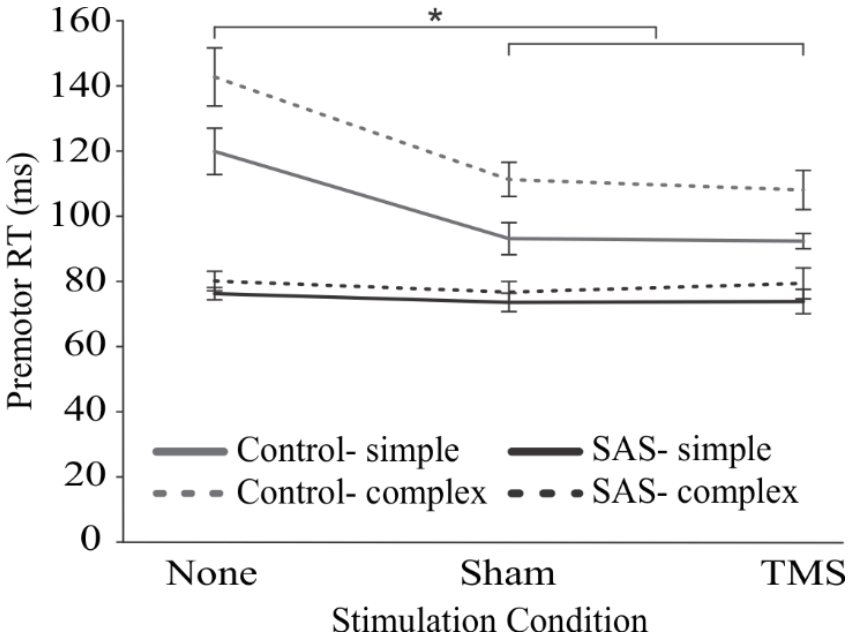

Figure 5. Mean (SE) premotor reaction time (RT) across TMS and acoustic stimulation conditions in Experiment 2. Premotor RT in the no stimulation (None), sham TMS, and real TMS conditions are shown as a function of acoustic stimulation condition (Control vs. SAS), and task complexity (simple vs. complex). Asterisks (*) denote significant ($p > .05$) differences between TMS conditions in the control conditions (for both the simple and complex tasks), as there were no significant differences between the SAS conditions.

5.3. Task performance. Analysis revealed that in the simple task there were no significant differences between conditions for the duration of the key press ($p > .05$), indicating that participants were performing a similar movement regardless of the presence of a SAS or TMS. Similarly, in the complex task there were no significant differences in the duration of key press 1, 2 or 3, as well as no significant differences between conditions for IRI-1 or IRI-2 ($p > .05$). These results indicate that participants were also performing similar movements for the complex task across all experimental conditions.

5.4. EMG activity. For the simple task, analysis revealed that there were no significant differences between any of the conditions for initial ECR burst duration, $p > .05$, whereas for initial ECR peak amplitude there was a significant main effect of imperative stimulus, $F(1, 5) = 9.421$, $p = .028$, $\eta^2_p = .653$. This indicated that the peak amplitude of the initial ECR burst was significantly greater in startle trials (0.197 mV, SD = 0.12) than in control trials (0.106 mV, SD = 0.049). Similarly, in the complex task, it was shown that there were no significant differences between any of the conditions for initial ECR burst duration ($p > .05$), but there was a significant main effect of imperative stimulus for initial ECR peak amplitude, $F(1, 5) = 10.232$, $p = .024$, $\eta^2_p = .672$. Again, the peak amplitude of the initial ECR burst was significantly greater in startle trials (0.165 mV, SD = 0.11) than in control trials (0.092 mV, SD = 0.051).

5.5. SCM activity. The percentage of SAS trials where an SCM response was elicited in the simple movement condition was 86% (SD = 27) in trials without TMS, 83% (SD = 26) in trials with real TMS and 94% (SD = 14) in trials with sham TMS. For the complex movement condition, the percentage of SAS trials where an SCM response was elicited was 89% (SD = 15) in trials without TMS, 83% (SD = 26) in trials with real TMS and 86% (SD = 27) in trials with

sham TMS. Analysis revealed that there was no significant difference in the percentage of SCM responses elicited across conditions, $p > .05$.

6. Discussion

The purpose of the present experiments was to investigate cortical involvement in the neural pathway underlying the RT-speeding effects of startle. Specifically, sub-threshold TMS was applied over M1 early in the RT interval for a complex movement in order to determine if TMS would result in facilitation of startle RT in the same manner as control RT, providing evidence for a role of cortex in the StartReact effect. Conflicting evidence regarding cortical involvement in the StartReact effect has led to the generation of two competing hypothesis for the RT speeding effect of a SAS: 1) the sub-cortical storage hypothesis (Carlsen et al., 2004a; Nonnekes et al., 2014; Valls-Solé et al., 1999) and 2) the cortical storage hypothesis (Alibiglou & MacKinnon, 2012; Carlsen et al., 2012; Maslovat, Carter, et al., 2014). Experiments using sub-threshold TMS to distinguish between these hypotheses have shown that there was no effect of TMS on startle RT regardless of the time of TMS application (Smith & Carlsen, 2017; see Appendix B). The authors suggest that while these results appear to support the sub-cortical storage hypothesis, it is also possible that a floor effect occurred in the startle condition, as the startle RTs across all experimental conditions were similar. To address this limitation, the present experiment employed two movements of greater complexity, which have been shown to lead to increases in both control and startle RT (Klapp, 2003; Maslovat, Klapp, et al., 2014). The results of the present experiment showed that despite increasing timing complexity, startle RT was not significantly slower than in simple movements. Furthermore, there was no effect of either real or sham TMS on startle RT. While these results may indicate a limited role of the

cortex in the StartReact effect, this may still be the result of an RT floor effect as has been posited previously.

In both experiments, presentation of a SAS resulted in significant reductions in RT and consistent SCM activation, while task performance and movement kinematics were similar between control and startle trials. Peak amplitude of the initial EMG burst of the wrist extensor was greater in SAS trials for both experiments, which is not an uncommon finding, and is believed to be due to a SAS resulting in a larger initiation signal, or to summation between the voluntary command and the startle volley (Carlsen et al., 2013; Siegmund et al., 2001). Initial wrist extensor EMG burst duration was also significantly shorter in startle trials than in control trials in Experiment 1, while the duration of the first key press was longer. Similar results were not seen in Experiment 2, suggesting that these differences likely represent a chance occurrence. As such, the results of the present experiments represent a typical StartReact effect (Valls-Solé et al., 1999), allowing for an investigation of the neural mechanism underlying this phenomenon. Experiment 1 was designed to increase RT, while still limiting the timing complexity of the movement. It has previously been shown that a three-key press movement results in significantly slower startle RT than a single key press movement, with slower RT when the timing between key presses is non-isochronous than when it is isochronous (Maslovat, Klapp, et al., 2014). While the goal of the present experiment was to increase startle RT, increasing timing complexity increases cerebellar contributions to the movement (Ivry & Keele, 1989); thus, isochronous timing was used to maximize the cortical nature of the task. However, the results of Experiment 1 showed startle RTs that were approximately the same as those seen in previous experiments employing a simple movement. Also similar to previous research, there was no significant effect of either real or sham TMS on startle RT, despite both forms of TMS reducing

RT in control trials (see Figure 4). These results appear to provide further support for the sub-cortical storage hypothesis; however, the startle RTs suggest that a floor effect may again have been reached, despite using a more complex movement, limiting the ability to draw conclusions on the mechanism underlying the StartReact effect.

In Experiment 1, only participants who exhibited ICF were allowed to participate in the entirety of the experimental protocol, resulting in a significant ICF effect elicited across participants as a whole (see Figure 3); though this greatly limited the final sample size, as fewer than 35% of participants met all inclusion criteria. This ICF inclusion criterion was based on the thought that a direct facilitatory effect of sub-threshold TMS on MEP amplitude must be seen in order to infer any possible cortical effects of TMS on RT in control trials. However, further examination of this phenomenon has shown that there does not appear to be any difference in the RT patterns in response to sub-threshold TMS between those who exhibit ICF and those who do not (Smith & Carlsen, 2017; see Appendix B). These results provide evidence that the facilitation of MEPs and RT by sub-threshold TMS may not share a common underlying mechanism, yet, due to the small sample size further research is warranted to definitively rule out this possibility. Furthermore, ICF has been shown to be highly variable both between and within subjects (Hermsen et al., 2016; Orth et al., 2003), with participants exhibiting ICF at inter-stimulus intervals ranging from 6 – 500 ms (Du, Summerfelt, Chiappelli, Holcomb, & Elliot Hong, 2014). As such, the use of ICF as an inclusion criterion is likely not only unnecessary, but may also introduce significant, unwarranted experimental constraints.

Due to the limitations of Experiment 1, a second experiment was conducted to investigate cortical involvement in the RT-speeding effects of a SAS. To maximize the likelihood of increasing RT in startle trials, participants performed a three-key press movement with non-

isochronous timing (Maslovat, Klapp, et al., 2014), as well as a single key-press movement to allow direct comparison of a simple and complex movement within individuals. In addition, the presence of ICF was not used as an inclusion criterion, but ICF testing was included in order to further examine the commonality of the mechanism underlying sub-threshold TMS facilitation of MEPs and RT. While the complex task resulted in slower RT in control trials, there was again no significant increase in startle RT in Experiment 2 (in contrast to results reported by Maslovat, Klapp, et al., 2014), and no difference between TMS stimulation conditions in startle trials (see Figure 5). Similar to Experiment 1, these results appear to suggest that the cortex is not involved in the neural mechanism underlying the StartReact effect, though it is still possible that a floor effect of RT was reached, limiting the utility of manipulations aiming to reduce startle RT. The use of a stop-signal task has been shown to result in significant increases in RT under startle conditions (Drummond, Cressman, & Carlsen, 2016), providing a possible task for future research employing sub-threshold TMS within a startle paradigm.

While the present experiment was primarily designed to investigate the effect of sub-threshold TMS on startle RT, the results of control conditions provide a final note of interest. When sub-threshold TMS is applied over M1 early in the RT interval, it results in significant facilitation of RT (Pascual-Leone et al., 1992; Sawaki, Okita, Fujiwara, & Mizuno, 1999; Terao et al., 1997), and this facilitation has been shown to be due to an additive effect of intersensory facilitation and a direct effect of TMS on the cortex (Smith & Carlsen, 2017; see Appendix B). In Experiments 1 & 2 both real and sham TMS resulted in reductions in control RT, with no difference between real and sham TMS in either experiment. This result was seen in Experiment 1, despite all participants exhibiting ICF, further supporting the notion that the facilitatory effects

of sub-threshold TMS on MEPs and RT may work via differing mechanisms. However, this interpretation should be treated with caution as both Experiments included a limited sample size.

It has been shown that increasing the timing complexity of a multiple component movement results in increases in simple RT (Klapp, 2003; Klapp, Wyatt, & Lingo, 1974; Maslovat, Klapp, et al., 2014); however, the mechanism underlying this effect is not fully understood (Maslovat, Chua, Klapp, & Franks, 2016; Maslovat, Klapp, et al., 2014). It has been suggested that these increases in RT are due to the inability to pre-program the timing aspect of a movement, as well as initiation processes beginning later and occurring at a slower rate for movements with greater timing complexity (Kennefick, Maslovat, Chua, & Carlsen, 2016; Maslovat et al., 2016). Based on this, the lack of RT facilitation for the complex movement in control trials may have been due to the timing of TMS application being too early in the RT interval, as the time was chosen based on the results of experiments employing a simple task. In a more complex task, where initiation processes begin later and accumulate at a slower rate, TMS may need to be applied later in the RT interval for sub-threshold TMS to exert its facilitatory effect. While this can explain the control results of the complex movements in Experiments 1 & 2, there was also no difference in RT between real and sham TMS for the simple task used in Experiment 2 (see Figure 5). As the RTs for the simple movement were similar to those seen in previous experiments, it is unlikely that the lack of an effect was due to the timing of TMS application. It has been shown that TMS over vertex does not interfere with processing occurring within M1; however, it is possible that vertex stimulation led to activation within other brain regions, such as SMA, which may have enhanced movement preparation/execution. These limitations, combined with the small sample size of participants in the present experiment, warrant further research to corroborate these results.

In conclusion, the results of the present experiment suggest that the cortex may not be involved in the neural pathway underlying the StartReact effect, as sub-threshold TMS did not result in facilitation of startle RT. However, despite employing tasks with greater timing complexity, RT in both Experiments 1 & 2 was not slower than that for a simple movement. As such, it is possible that the results of startle conditions represent a floor effect, and humans are not capable of responding any faster, even if the cortex plays a role in mediating startle-triggered movements. It has been shown that startle RT is significantly slower for a stop-signal task (Drummond et al., 2016), providing a possible task for future research aiming to investigate the neural mechanism underlying the StartReact using sub-threshold TMS. In contrast, the increased activation provided by a SAS may be too great for the added activation from sub-threshold TMS to have a significant effect in startle conditions, necessitating the generation of an alternative method of investigating cortical involvement in the RT-speeding effects of startle.

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Chapter III: General Discussion

It is well-established that RT in a simple RT paradigm is significantly shorter in response to a SAS than to a control imperative stimulus, due to the SAS involuntarily triggering the release of a prepared motor program (Carlsen et al., 2004a; Carlsen et al., 2012; Valls-Solé et al., 1999). While the StartReact effect has been widely studied, cortical involvement in the neural mechanism underlying this phenomenon remains widely debated in the motor control literature (Carlsen et al., 2012; Nonnekes et al., 2015; Nonnekes et al., 2014). As such, the aim of this thesis was to investigate the involvement of the cortex in the neural pathway underlying the RT-speeding effects of a SAS through the use of sub-threshold TMS. Previous research employing this paradigm found that a floor effect of RT may have been reached in startle trials, limiting the ability to draw conclusions regarding the mechanism underlying the StartReact effect (Smith & Carlsen, 2017; see Appendix B). Despite the use of movements with increased complexity in both of the present experiments, there were no significant increases in startle RT, and no differences between TMS conditions in startle trials. These results suggest the cortex may not be involved in the involuntary initiation of movements by a SAS; however, it is also possible that the results of the present experiment again represent a floor effect, and humans are simply not capable of responding any faster.

The lack of an increase in startle RT in the present experiment significantly limits the ability to draw conclusions with respect to the neural pathway underlying the RT-speeding effects of a SAS. It is well-established that under particular conditions (i.e. multiple component movements) simple RT increases along with the complexity of the required movement (Klapp, 2003). Maslovat et al. (2014) provided evidence that this is also seen in startle trials; increasing timing complexity of a multiple component movement (three-key press) led to increases in

startle RT. The present experiment employed the same tasks as those used by Maslovat et al. (2014), but increases in RT were only seen in control trials. The reason for this difference in results is unclear; however, it is possible that in individuals who are very susceptible to startle (i.e., exhibit SCM activation in response to a SAS more than 90% of the time) the increased activation provided by the SAS is so great that rapid RTs are achieved regardless of movement complexity. This hypothesis is supported by the results of Experiment 2, where two participants exhibited a relatively lower proportion of SAS trials where an SCM burst was elicited (67% & 75%), and these two participants had startle RTs that were ~16 ms slower for the complex movement than the simple movement. In contrast, in those individuals who exhibited SCM activation on more than 90% of SAS trials there were no differences in startle RT between the simple and complex movements. While these results provide a possible explanation for the lack of RT differences in the present experiment, a similar pattern was not seen in Experiment 1, and the sample size in Experiment 2 was too small to make any definitive conclusions. It is also possible that the results of Maslovat et al. (2014) may have represented a chance finding, as this is the only study to date that has investigated increased startle RT for multiple component movements. Whatever the reason for the startle RT results obtained in the present experiment, further research is warranted to investigate the involvement of the cortex in the StartReact effect, as well the effect of movement complexity on startle RT.

A second significant limitation encountered in the present experiment was the intra-individual variability of responses to paired-pulse TMS in Experiment 1. It is widely accepted that the presentation of a sub-threshold TMS pulse 6-30 ms prior to a supra-threshold pulse results in facilitation of the resulting MEP (Chen, 2004; Hanajima & Ugawa, 2012; Kujirai et al., 1993; Ni & Chen, 2011; Rossini et al., 2015). It has also been shown that 25 pulses provides a

reliable measure of ICF (Chang et al., 2016; Goldsworthy, Hordacre, & Ridding, 2016). Despite employing ICF parameters similar to those used in previous studies, and delivering 25 pulses of both single and paired-pulse TMS in the ICF testing blocks, only a small percentage of participants exhibited ICF in the present experiment. As the presence of ICF in these blocks was used as an inclusion criterion in Experiment 1, this resulted in the exclusion of 61% (11/18) of participants from participating in the remainder of the experimental protocol. While achieving ICF with a conditioning pulse applied 6-30 ms prior to a test pulse is often stated as fact, research investigating the variability of paired-pulse TMS measures provides evidence that these results are highly variable (Boroojerdi et al., 2000; Hermsen et al., 2016; Orth, Snijders, & Rothwell, 2003; Wassermann, 2002; Wassermann & Zimmermann, 2012). For example, Wassermann (2002) found that while many individuals exhibited ICF at inter-stimulus intervals of 10 & 15 ms, a portion of individuals exhibited no modulatory effects of TMS, while others exhibited significant intracortical inhibition. The significant variability seen with paired-pulse paradigms is believed to be due to a combination of experimenter error, within-subject factors, and spontaneous cortical excitability fluctuations and spinal desynchronization (Jung et al., 2010; Wassermann, 2002). While these findings indicate that the results obtained in the present experiment are not unprecedented, the variability of ICF effects are a significant limitation when the presence of ICF is being used as an inclusion criterion. This was included as previous research provided evidence that the facilitatory effect of sub-threshold TMS on MEPs and RT may be due to a similar underlying neural mechanism (Smith & Carlsen, 2016). However, further investigation of this phenomenon (Smith & Carlsen, 2017; see Appendix B), in conjunction with the results of the present experiment, suggest that the mechanism underlying these TMS effects are likely due to separate mechanisms. Based on this, the presence of ICF in

the ICF testing blocks was not included as an inclusion criterion in Experiment 2, greatly increasing the number of eligible participants.

A third limitation of the present experiment was the lack of a significant RT difference between the real and sham TMS conditions in control trials. Sub-threshold TMS was chosen to investigate cortical involvement in the RT-speeding effects of startle because it has been shown to result in RT reductions in control trials due in part to a direct effect of TMS on the cortex (Smith & Carlsen, 2017; see Appendix B). The facilitatory effect of sub-threshold TMS on RT has been found in various movements including finger abduction (Nikolova, Pondev, Christova, Wolf, & Kossev, 2006), elbow flexion (Pascual-Leone, Valls-Sole, et al., 1992) and wrist extension (Terao et al., 1997). In these experiments RT-facilitation was seen when TMS was applied 90-150 ms prior to participants' control RT (Hashimoto et al., 2004; Pascual-Leone, Valls-Sole, et al., 1992; Terao et al., 1997). Based on these results, the window for TMS facilitation in the present experiment, where participants' control RT was approximately 150 ms, would have been from 0 ms to +60 ms with respect to the go-signal. The timing of TMS application in the present experiments, +30 ms, falls within this window for RT facilitation; however, there was no significant facilitatory effect of TMS beyond that of intersensory facilitation. As this window for TMS facilitation is based on the results of experiments employing simple tasks, it is possible that TMS was applied too early to elicit a facilitatory effect of TMS on RT for more complex movements. This is supported by research employing TMS to investigate corticospinal excitability during a task similar to those used in the present experiments. Kennefick et al. (2016) found that for movements with increased timing complexity initiation-related activation increases at a slower rate, and also begins slightly later than for complex movements. As the time of TMS application in the present experiment was relatively

early in the RT window of expected RT facilitation for simple movements, it is quite possible that it was too early for any effects to be seen for a movement with increased timing complexity. This lack of a direct effect of TMS on RT further limits the ability to draw conclusions regarding the mechanism underlying the StartReact effect from the present experiment. If TMS had resulted in facilitatory effects on startle RT, the lack of a direct effect on the cortex in control trials would have left the locus of these RT differences unclear.

While the limitations of the present experiment diminish the ability to draw conclusions with regards to the original research question, the rationale underlying this question provides a discussion of interest. The primary goal of this thesis was to determine if the cortex, specifically M1, plays a role in the RT-speeding effects of a SAS, providing evidence for the cortical storage hypothesis. In the literature there is debate between researchers in support of the cortical storage hypothesis (Carlsen et al., 2012; Stevenson et al., 2014), and researchers in support of the sub-cortical storage hypothesis (Honeycutt & Perreault, 2012; Nonnekes et al., 2015), with the debate centering on whether or not the cortex is involved in the StartReact effect. Both of these hypotheses aim to provide an explanation for the neural pathway underlying the RT-speeding effects of a SAS, generally assuming that all movements are initiated by a SAS via the same mechanism. In contrast, it has recently been suggested that there may not be just one mechanism that underlies all instances of the StartReact effect, rather the underlying pathway may vary depending on the cortical and sub-cortical contributions to the movement. Evidence for this hypothesis comes from a study employing supra-threshold TMS in a startle paradigm where participants were required to perform both flexion and extension movements (Carlsen, Maslovat, & Hajj, 2016). Preliminary results showed that the RT delays induced by TMS in both control and startle trials were smaller for flexion movements than extension movements, providing

evidence for differing cortical and reticular contributions to the preparation and initiation of these movements. This hypothesis has also been investigated using EMG-EMG coherence analysis, which can elucidate cortical and reticular contributions to movement based on the differing EMG frequency bands associated with these differing neural inputs (Grosse & Brown, 2003). When participants performed focal and postural movements with the same muscle, there were different neural contributions to the movements in both control and startle conditions depending on whether the muscle was acting focally or posturally (Leguerrier, Maslovat, Bui, & Carlsen, 2016). Taken together, these results provide evidence that the neural pathway underlying the involuntary initiation of prepared movements by a SAS may have differing contributions of cortical and sub-cortical structures depending on the movement being performed. With respect to the present experiment, if increasing timing complexity had resulted in increased startle RT, it is possible that sub-threshold TMS would have resulted in decreased RT compared to the no TMS condition. While this could have been taken as evidence for the cortical storage hypothesis, generalizing the results of such a complex movement to all movements may have been unwarranted. Rather, within the cortical/reticular contribution proposal, the expected results could only have been taken as evidence that the cortex is involved in the StartReact pathway underlying complex, multiple component movements.

In conclusion, the results of the present experiment appear to suggest that the cortex may not be involved in the neural mechanism underlying the StartReact effect; however, due to several limitations no definitive conclusions can be made based on the present results. The primary limitation encountered was the lack of an increase in startle RT when the timing complexity of the movement was increased. It has been shown that startle RT is significantly slower in a stop-signal task (Drummond, Cressman, & Carlsen, 2016), providing a possible

method for researchers employing sub-threshold TMS within a startle paradigm to investigate the StartReact effect. However, due to the limitations encountered with this paradigm, an alternative method of investigating the neural mechanism underlying the RT-speeding effects of startle may be more fruitful. This is especially true when considering the proposal that there may not be a single mechanism underlying the involuntary initiation of prepared movements by a SAS, rather the initiation mechanism depends on the cortical and reticular contributions to different movements. As the neural mechanism underlying the RT-speeding effects of startle remains up for debate, further research is warranted to fill this gap in the motor control literature, and allow researchers a clearer understanding of the results of research employing a startle paradigm.

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Appendix A: Hypothesized neural mechanisms underlying the StartReact effect

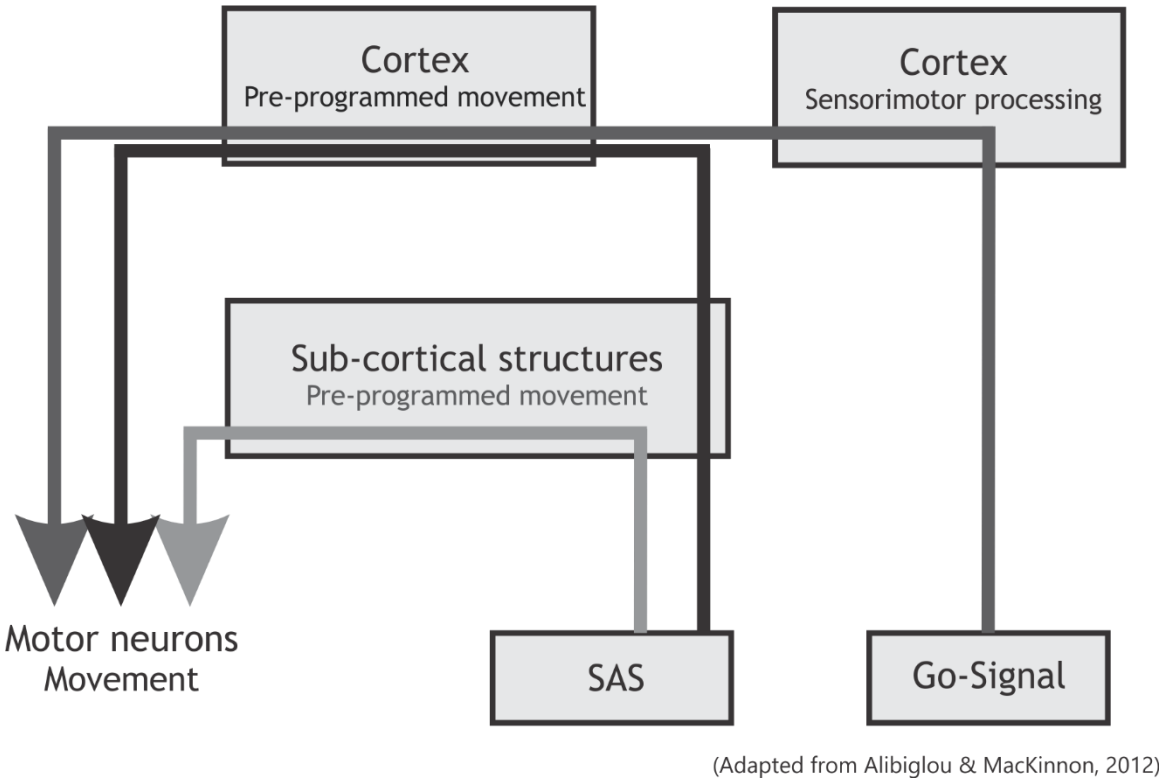

Figure A-1. Competing hypotheses for the neural mechanism underlying the StartReact effect. The black arrow represents the cortical storage hypothesis, the light grey arrow represents the sub-cortical storage hypothesis, and the dark grey arrow represents the pathway underlying the voluntary initiation pathway.

Appendix B: Manuscript of previous experiments

(In review: Journal of Neurophysiology)

Subthreshold transcranial magnetic stimulation applied after the go-signal facilitates reaction time under control but not startle conditions

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Author Contributions.

A.N.C. contributed to the conception and design of the study, the acquisition, analysis and interpretation of data, and critically revising the manuscript. V.S. contributed to the design of the study, the acquisition, analysis and interpretation of the data, and the drafting and revision of the manuscript.

Abstract

The presentation of a startling acoustic stimulus (SAS) in a simple reaction time (RT) task significantly reduces RT due to the involuntary early initiation of a prepared movement; however, the underlying neural mechanism remains unclear. It has been proposed that a SAS triggers a cortically stored motor program by involuntarily increasing initiation-related activation. Sub-threshold transcranial magnetic stimulation (TMS) can be used to investigate cortical processes, as it increases cortical excitability for 6-30ms and significantly reduces RT. The purpose of the present experiments was to determine if application of sub-threshold TMS over motor cortex in close temporal proximity to a SAS would facilitate startle RT in the same manner as control RT, providing evidence for cortical involvement in startle-elicited RT. Participants completed a simple RT task requiring targeted wrist extension in response to an auditory go-signal, which was randomly replaced by a SAS on 25% of trials. On a subset of trials sub-threshold TMS was applied 30ms following the go-signal in control trials, or at -15, 0, +15 or +30ms with respect to the SAS in startle trials. In all three experiments sham and real TMS significantly reduced RT in control trials, with real TMS having a larger effect, but there was no effect of either real or sham TMS on startle-elicited RT. These results suggest that either prepared movements can be stored and initiated via subcortical structures with limited cortical involvement, or that startle results in a floor effect on RT.

New and Noteworthy

In this experiment, sub-threshold TMS was employed to investigate cortical involvement in startle-facilitated RT. In control trials, TMS reduced RT due to an additive effect of intersensory facilitation and a direct effect on the cortex. However, TMS had no effect on startle RT. While

these results appear to suggest the cortex is not involved in the RT-facilitation effects of startle, it is also possible that startle results in a floor effect of RT.

Keywords: Intracortical facilitation, startle, reaction time

Introduction

It is well established that the presentation of a startling acoustic stimulus (SAS) in conjunction with the imperative go-signal in a simple reaction time (RT) task leads to significant reductions in RT that have been attributed to involuntary triggering of the prepared movement (Carlsen, Maslovat, & Franks, 2012; Valls-Solé, Kumru, & Kofler, 2008). While it was originally suggested that this RT facilitation may simply be due to the summation of the general startle reflex and the voluntary response, there is ample evidence that the RT facilitation is due to the involuntary triggering of a prepared movement (see Carlsen, Maslovat, et al., 2012; Marinovic & Tresilian, 2016 for reviews). For example, Valls-Solé et al. (1999) had participants perform either wrist flexion or extension movements, and found no difference between the EMG patterns in control or startle trials. Furthermore, the EMG patterns in the flexion and extension trials were so similar that the EMG results were collapsed across these movements. It has also been shown that a SAS presented up to 1.5 s before the go-signal results in similar short latency response initiation as a SAS presented at the go-signal, indicating the prepared movement is being involuntarily triggered, as participants would not voluntarily initiate movements this far in advance of the go-signal (Carlsen & MacKinnon, 2010; MacKinnon, Allen, Shiratori, & Rogers, 2013). This SAS-related involuntary response triggering and facilitation of RT has been termed the StartReact effect (Carlsen, Chua, Inglis, Sanderson, & Franks, 2004b; Valls-Solé et al., 1999). While the use of a SAS has allowed researchers to investigate the preparation and initiation mechanisms underlying the production of various movement types in both healthy (Castellote, Kumru, Queralt, & Valls-Solé, 2007; MacKinnon et al., 2007; Oude Nijhuis et al., 2007; Siegmund, Inglis, & Sanderson, 2001) and clinical populations (Carlsen, Almeida, &

Franks, 2013; Carlsen, Maslovat, et al., 2012; Honeycutt & Perreault, 2012; Nonnekes et al., 2014), the mechanism behind the RT shortening effect of a SAS remains widely debated.

Due to the speed of responses following a SAS (as fast as 65 ms), Valls-Solé et al. (1999) suggested that there was not adequate time for the involvement of a transcortical pathway, as the minimum sensorimotor conduction time for wrist responses is approximately 55 ms. This led to the proposal that a SAS bypassed the usual cortical pathway and involuntarily triggered the release of a subcortically stored motor program (Carlsen, Chua, Inglis, Sanderson, & Franks, 2004a; Valls-Solé et al., 1999). Evidence for this notion was provided by the observation that the fastest RTs in response to a SAS occurred when a burst of EMG was seen in the sternocleidomastoid (SCM) (Carlsen, Dakin, Chua, & Franks, 2007), which is associated with strong activation in the brainstem structures responsible for producing the startle reflex (Brown et al., 1991). Furthermore, it was recently shown that RT was normalized in response to a SAS in patients with hereditary spastic paraplegia, who typically show delayed RTs due to degeneration of the corticospinal tract. This result was attributed to the voluntary response being conveyed by the intact reticulospinal system (Nonnekes et al., 2014).

While these results support the notion that the fast RTs observed in response to a SAS are primarily mediated by pathways that bypass cortical processing, recent research employing transcranial magnetic stimulation (TMS) has provided evidence that the cortex may nevertheless play an important role in the StartReact effect. The application of high-intensity single-pulse TMS can be used to interrupt voluntary cortical drive for a short time (Fuhr, Agostino, & Hallett, 1991), which leads to decreased excitability of the cortex – a phenomenon known as the "cortical silent period." Alibiglou and MacKinnon (2012) applied high intensity TMS over contralateral primary motor cortex (M1) during a simple RT task and showed significant delays in RT.

Importantly, the cortical silent period protocol also led to delayed RTs in SAS trials, implying that M1 must play a role in the pathway underlying the RT facilitation effects of startle (see also Stevenson et al., 2014). Based on these findings, it was proposed that a SAS may lead to the early and involuntary triggering of a cortically stored motor program via a subcortically mediated increase in initiation-related activation processes (Carlsen, Maslovat, et al., 2012). This proposal is based on a neural accumulation model where preparation and initiation are separate processes, with preparation involving increasing activation to a level below movement threshold, and initiation involving the input of additional activation to surpass this threshold (Hanes & Schall, 1996). It was argued that the exceptionally fast RTs observed, even though cortex is involved, are a result of the SAS directly activating response initiation processes via an ascending reticulo-thalamo-cortical pathway, allowing threshold to be reached without engaging the usual cortical information processing (Carlsen, Maslovat, et al., 2012).

Despite evidence for cortical involvement in the StartReact effect from the cortical silent period protocol, it was suggested that these results may have been due to residual reduced reticulospinal excitability, as high intensity supra-threshold TMS leads to strong descending drive in both the cortico-spinal and cortico-reticular pathways (Fisher, Zaaimi, & Baker, 2012). One possible alternative method to investigate the brain areas involved in the StartReact effect involves the use of sub-threshold TMS. It has been shown that applying a sub-threshold TMS pulse 6-30 ms prior to a supra-threshold test stimulus leads to significant facilitation of the motor evoked potential (MEP). This facilitation is believed to occur as a result of activation of a low-threshold facilitatory circuit within the cortex, and is known as intracortical facilitation (ICF) (Kujirai et al., 1993). There are no discernable descending volleys in the spinal cord following the sub-threshold conditioning pulse (Di Lazzaro & Rothwell, 2014), supporting its use in

examining the role of the cortex in the StartReact effect without the confounding effects of descending reticulo-spinal activity.

When sub-threshold TMS is applied over contralateral M1 early in the RT interval in a simple RT paradigm, it leads to significant RT facilitation (Pascual-Leone et al., 1992; Soto, Valls-Sole, & Kumru, 2010). This may be due to TMS adding extra energy to M1, facilitating the transfer of a motor program to the neurons responsible for task execution (Pascual-Leone et al., 1992). However, it has also been argued that this RT facilitation may simply be due to speeded sensory effects, such as intersensory facilitation (Nickerson, 1973) and/or stimulus intensity (Woodworth, 1938), as an auditory click and vibration of the scalp also accompany TMS pulses (Terao et al., 1997). This suggests that in order to employ sub-threshold TMS in RT experiments a sham condition is necessary to control for the non-specific effects of TMS.

The purpose of the present study was to investigate if sub-threshold TMS applied over M1 early in the RT interval following a SAS would result in facilitation of startle elicited RT. In a first experiment, sub-threshold TMS was applied over M1 30 ms following the control go-signal or 15 ms following the SAS. A sham condition consisting of a matched "click" noise was also presented at matched latencies. It was hypothesized that sub-threshold TMS would lead to RT facilitation in both control and SAS trials, providing evidence for cortical involvement in the pathway of startle-triggered movements. A second experiment was performed to control for the timing of the TMS with respect to response initiation processes. Additionally, in this control experiment the sham condition used real TMS applied over M1, but with a different induced current direction that has been shown to not lead to ICF. This was included to control for the entirety of the sensory experience that accompanies TMS (Duecker & Sack, 2015), allowing for the investigation of the mechanism underlying the RT facilitation of sub-threshold TMS. Finally,

a third experiment was conducted to further control for the timing of TMS with respect to the accelerated initiation processes seen in response to a SAS.

2. Experiment 1 Methods

2.1. Participants. Fifteen adults with normal or corrected to normal vision and no obvious upper body abnormalities participated in this experiment. Prior to beginning the experiment, all participants were required to fill out a TMS safety questionnaire and only those whose answers indicated they had no contraindications to TMS were allowed to participate (Rossi, Hallett, Rossini, & Pascual-Leone, 2011). The data of one participant was excluded from the analysis as this participant did not show consistent SCM activation in response to a SAS, which was used as an indication of a startle reaction (Carlsen, Maslovat, Lam, Chua, & Franks, 2011). This resulted in data from fourteen participants (6 male, 8 female; M_{age} = 23 years, SD = 6) being included in the final analysis. All participants provided written informed consent before participating, and the experiment was approved and conducted in accordance with the ethical guidelines set by the Health Sciences and Science Research Ethics Board at the University of Ottawa, and conformed to the latest revision of the Declaration of Helsinki.

2.2. Apparatus and task. Participants were seated in a padded chair approximately 1.5 m from a 24-inch LCD computer screen with their right arm abducted approximately 30^o and flexed approximately 90^o at the elbow. The right forearm rested parallel to the floor in a custom-made manipulandum such that the palm faced inwards with the axis of rotation of the wrist aligned with the axis of rotation of the manipulandum. In order to restrict movement to only the wrist, the forearm was fastened in place by Velcro straps placed just distal and proximal to the elbow and wrist joints, respectively. Participants were required to perform a 20^o ballistic wrist extension in response to an auditory go-signal starting from a neutral position (wrist neither

flexed nor extended). Participants were instructed to react to the go-signal making the requisite movement as quickly and accurately as possible. Feedback regarding RT and movement accuracy were displayed on the monitor at the end of each trial. To encourage quick reactions, a points system rewarded participants for fast RTs (typical points threshold was ≤ 150ms) and penalized them for slow RTs; however, these points were only provided as an incentive and were not analyzed.

2.3. Experimental Procedure. All trials began with the words 'Get Ready!' being displayed on the computer screen for 1000 ms, followed by an auditory warning signal (100 ms, 200 Hz, 80 dB). After the warning signal, the screen went blank and following a random variable foreperiod (1500 to 2500 ms; uniform distribution) the auditory imperative stimulus (IS) (82 dB, 25 ms, 1000 Hz sine wave) was presented. Both the warning signal and the IS were generated with digital to analog hardware (National Instruments PCIe-6321), and amplified and presented by a loudspeaker (MG Electronics M58-H, frequency response 300 Hz - 11 kHz, rise time <1 ms). The loudspeaker was located 30 cm behind the participant, measured on an individual basis from the opening of their auditory canal. After completion of the movement, feedback regarding the just completed movement (RT and target accuracy) and points earned/lost for the trial were displayed for 3000 ms until the beginning of the next trial. A customized LabVIEW (National Instruments Inc.) program controlled the timeline for each trial, as well as the display of information to the participant.

One block of 10 practice trials was completed to allow participants to become comfortable with the task. The practice trials were identical to experimental trials with the exception that there were no TMS or SAS stimuli. Participants then completed five blocks of 24 experimental trials (total 120 trials). Each block contained 14 trials with the control IS, 2 control

IS trials with TMS, 2 control trials with sham TMS, as well as 2 trials where the IS was replaced by a SAS (120 dB, 25 ms, white noise waveform), 2 SAS trials with TMS, and 2 SAS trials with sham TMS. This number of trials was chosen to ensure participants were not exposed to the SAS too frequently (30/120 trials) (Carlsen et al., 2011), but also to ensure that there were sufficient TMS trials, which increases the likelihood of obtaining a representative sample mean and minimizes within session variance (Orth, Snijders, & Rothwell, 2003). Acoustic stimulus intensities were confirmed using a precision sound level meter (Cirrus Research model CR:162C, "A" weighting, impulse setting) at a distance of 30 cm from the loudspeaker. Trial order was controlled by a computer and was pseudorandomized such that a SAS was never presented on the first two trials of a block, and there was never a SAS presented on two consecutive trials.

2.4. Recording equipment. Surface EMG was collected from the superficial muscle bellies of the right ECR, right flexor carpi radialis (FCR), and left SCM using bipolar preamplified (gain = 10) surface electrodes (Delsys Bagnoli DE-2.1) connected via shielded cabling to an external amplifier (Delsys Bagnoli-8). These electrodes were placed parallel to the muscle fibers and attached to the skin using double-sided adhesive tape. In addition, a grounding electrode (Dermatrode HE-R) was placed on the right lateral epicondyle. To minimize electrical impedance all electrode sites were cleaned with abrasive skin prepping gel and alcohol wipes prior to attachment. Wrist angular displacement data was collected with a potentiometer attached to the axis of rotation of the manipulandum. Raw band-passed (20- 450 Hz) EMG and potentiometer data were digitally sampled at 4000 (National Instruments PCIe-6321) using a customized LabVIEW program and stored for offline analysis. Data collection was initiated by

the computer for each trial 1000 ms prior to presentation of the imperative stimulus and continued for 3000 ms.

2.5. Transcranial magnetic stimulation. TMS was delivered using a Magstim 200^2 stimulator with a figure-of-eight coil (D70mm, Magstim Company Ltd, Whitland, Dyefield, UK) with a maximum stimulator output of 2.2 T. TMS was applied over the contralateral M1 representation of the extensor carpi radialis (ECR) of the right forearm. To locate this area, the midpoint between the nasion and inion, and the left and right preauricular notches was found, followed by measuring four centimeters laterally and one centimeter anteriorly and marking this location with a red grease pencil. To find the optimal area for generating MEPs from the ECR, test pulses were delivered to locations on the scalp near this mark in .5 cm steps to determine where the largest MEPs were found. All test pulses were delivered with the coil placed tangentially on the scalp and approximately perpendicular to the central sulcus, resulting in a posterior-anterior (PA) current direction. The location that elicited the largest MEPs in the ECR was then marked and saved using neuronavigation hardware and software (ANT Neuro Visor 2, Madison, WI). The resting motor threshold (rMT) for the ECR muscle was defined to the nearest 1% of stimulator output by finding the minimum intensity needed to elicit an MEP of 50 μV in five of ten trials (Rossini et al., 1994). TMS was delivered using 80% of each individual's rMT during the experiment, which has been shown to lead to ICF without producing an MEP (Kujirai et al., 1993). Throughout the experiment, the neuronavigation system was used to allow the researcher to maintain the coil position within 1 mm of the marked ECR location.

On TMS trials, the TMS was applied 30 ms following the IS. This timing of TMS presentation was chosen based on previous research investigating the facilitatory effect of sub-threshold TMS on RT where TMS was applied 90-150 ms prior to participants' control RT

(Hashimoto, Inaba, Matsumura, & Naito, 2004; Pascual-Leone et al., 1992; Terao et al., 1997). As control RTs of 120 – 140 ms are commonly seen in simple RT paradigms with an auditory go-signal, this suggests TMS should be applied in a window -30 to +50 ms with respect to the go-signal. Based on an accumulator model of neural activation, which indicates that initiation-related activation processes begin approximately 35 ms following the IS (Maslovat, Carter, Kennefick, & Carlsen, 2014), and a time window of 6 – 30 ms where ICF is generally seen (Rossini et al., 2015), TMS was applied just prior to the expected increase in neural activation to ensure the increase in activation from TMS would occur during initiation processes, and not before. On Sham TMS trials, an auditory "click" of the same sound profile and intensity of the TMS pulse was presented 30 ms following the IS from the speaker located behind the participant. The sham condition was included to control for the possible non-specific auditory effects of TMS that can influence RT (Terao et al., 1997). SAS trials that included TMS or sham TMS had these stimuli applied 15 ms following the presentation of the SAS. This timing was chosen because it has been shown that initiation-related activation processes accumulate faster (~33%) following a SAS (Maslovat, Carter, et al., 2014); thus, the time interval between TMS presentation and expected RT used in control conditions is not appropriate in startle conditions. Reducing this time interval by 33% suggests that TMS should be applied 60-100 ms prior to expected startle RT. In the present paradigm startle RT was expected to be ~80 ms, which would suggest application of TMS in a time window of 20 ms before the SAS to 20 ms following the SAS. As initiation processes begin approximately 20 ms following a SAS (Maslovat, Carter, et al., 2014), TMS was applied 15 ms following the SAS to maintain consistency across the control and startle conditions.

2.6. Data reduction and analysis. SAS trials with no discernible SCM activation (see below) were discarded, as this is considered to be a robust and reliable indicator of a sufficient startle reaction to elicit a StartReact response (Carlsen, 2015; Carlsen et al., 2007). SCM activation was defined as a burst of SCM EMG occurring within 50 to 120 ms of presentation of a SAS (Carlsen et al., 2011). One participant showed SCM activation in less than 60% of SAS trials and was thus excluded from further analysis. In addition, trials where participants were anticipating the go-signal (RTs $\leq$ 50 ms) or not paying sufficient attention to the task (RTs $\geq$ 300 ms) were discarded. Lack of SCM activation occurred in 32 trials, RTs that were too slow occurred in 12 trials, and RTs that were too fast occurred in 60 trials. This resulted in a 94% inclusion rate (1576/1680 trials).

EMG burst onset for all muscles (ECR, FCR and SCM) was defined as the point where rectified and filtered (25 Hz lowpass elliptic filter) EMG activity reached two standard deviations above baseline level (measured as the mean EMG in a 100 ms interval following the warning signal) and remained elevated for at least 20 ms. EMG offset was defined as the first point where EMG activity dropped below 20% of its maximal amplitude for that EMG burst. EMG traces were displayed on a computer monitor along with EMG onset and offset markers computed using a custom LabView algorithm (Hodges & Bui, 1996), and then manually adjusted to correct for any possible errors due to the strictness of the algorithm (i.e. marking a small muscle twitch prior to movement as EMG burst onset). Premotor RT was defined as the time between presentation of the imperative stimulus and initial EMG onset of the right ECR. Premotor RT was chosen as it is believed to represent central processing related to movement preparation following the go signal while excluding movement execution processes (Schmidt & Lee, 2011), and to allow for comparison with previous experiments investigating the effects of a

SAS on RT. Peak EMG amplitude was calculated as the largest EMG amplitude recorded within an interval of 100 ms following EMG burst onset (calculated from rectified and filtered EMG). The integrated area of the raw rectified EMG (iEMG) for the ECR muscle was calculated for six 30 ms windows beginning from EMG onset (0-30 ms, 30-60 ms, 60-90 ms, 90-120 ms, 120-150 ms, 150-180 ms). Finally, duration of the first ECR burst was defined as the time between EMG onset and offset in ECR.

2.7. Statistical Analysis. Shapiro-Wilk's tests of normality were performed for all dependent measures; parametric analyses were performed for all measures whose data was normally distributed, whereas non-parametric equivalents were performed for all measures whose data was not normally distributed. Dependent measures were analyzed using separate 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) repeated measures analysis of variance (RM ANOVA) to determine if there were any differences in premotor RT or EMG measures due to the presentation of a SAS, TMS, or sham TMS. To determine if TMS had any effect on the overall EMG profile iEMG values were analyzed using a 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) x 6 (Time: 1, 2, 3, 4, 5, 6) RM ANOVA. Greenhouse-Geisser corrected degrees of freedom were used to correct for any violations of sphericity. The significance value for all statistical tests was set at $p < .05$, and where appropriate, partial eta squared (η^2_p) is reported to provide an estimate of effect size. All significant differences were analyzed using a Tukey's Honestly Significant Difference (HSD) post-hoc test to determine the locus of the significant difference. All analyses were performed using the statistical software package SPSS 21 for Windows (IBM Inc., Armonk, NY, USA).

3. Experiment 2 Methods

3.1. Participants. 15 adults with normal or corrected to normal vision, no obvious upper body abnormalities and no contraindications to TMS (Rossi et al., 2011) participated in Experiment 2. Four participants did not demonstrate consistent SCM activation, and technical difficulties occurred during the testing of one participant, resulting in a final sample size of 10 participants (6 male, 4 female; M_{age} = 25, SD = 6). All participants provided written informed consent before participating. The experiment was conducted in accordance with the ethical guidelines of the Health Sciences and Science Research Ethics Board at the University of Ottawa, and conformed to the latest revision of the Declaration of Helsinki.

3.2. Experimental Procedure. The experimental procedure was similar to Experiment 1 with two main modifications: 1) the timing of TMS application in startle trials and 2) the type of sham condition used. To control for the possibility that any RT differences in Experiment 1 were due to the different timing of TMS application used between control and startle trials, TMS was applied 30 ms following the go-signal in both conditions in Experiment 2, despite falling slightly outside of the expected window for RT facilitation in startle trials. Previous research has highlighted the importance of including a sham condition that replicates *all* of the sensory aspects of TMS (Duecker & Sack, 2015); therefore, the sham TMS in Experiment 2 consisted of a TMS pulse being applied over the M1 representation of the right ECR, but with a latero-medial (LM) current direction rather than a PA direction (used in real TMS). This coil orientation elicits the same auditory and somatosensory experiences for the participant, but it has been shown that a conditioning stimulus inducing a LM current does not lead to ICF (Ziemann, Rothwell, & Ridding, 1996).

Prior to beginning testing, a block of 15 baseline MEPs was collected from the right ECR at 120% of rMT for each participant, as well as a block of 15 MEPs elicited using a conditioning

stimulus intensity of 80% of rMT presented 12 ms prior to the test stimulus (block order randomized). Stimulus delivery was performed using two Magstim 200^2 units combined using a Bistim^2 module (Magstim Company Ltd.). Data were collected for 300 ms at 10 kHz and saved for offline analysis. These trials were included as a confirmation that cortical excitability was actually modulated by the TMS parameters being used. Ensuring that ICF was not elicited in the sham TMS condition was not possible, as this would have required two TMS coils and complex experimental set-up.

Participants performed 20^o ballistic wrist extension movements in response to an auditory go-signal in the same manner as in Experiment 1. In contrast to the previous experiment, participants completed eight blocks of 16 trials, for a total of 128 trials. Four of the eight blocks consisted of 10 control trials, two control trials with TMS, two SAS trials, and two SAS trials with TMS. The other four blocks consisted of 10 control trials, two control trials with sham TMS, two SAS trials, and two SAS trials with sham TMS. The blocks were broken up in this manner due to the sham TMS condition requiring a 90^o rotation of the TMS coil, which cannot be done within a block as the experimenter was blind to trial order and participants would have felt the coil being rotated on their heads between trials. The order of blocks was randomly assigned and counterbalanced between participants. The remainder of the TMS, recording equipment and data reduction protocols were identical to those of Experiment 1. This resulted in exclusion of 38 trials due to lack of an SCM burst on startle trials, 9 trials due to RTs above 300 ms, 44 trials due to RTs below 50 ms, and 4 trials due to the TMS pulse not being delivered on a TMS trial, and an overall inclusion rate of 93% (1185/1280).

The statistical analyses performed were also identical to Experiment 1; with the addition of analyses of MEP amplitude in the paired pulse TMS and single pulse TMS trials. MEP

amplitude was defined as the greatest peak-to-peak amplitude recorded in a 30 ms window beginning 15 ms following the TMS pulse. MEPs were rejected if 1) root mean square EMG activity in the 50 ms preceding the TMS pulse exceeded twice the resting root mean square value for that trial (determined from a mean of the first 50 ms of EMG in the trial) or 2) if at any point prior to TMS application root mean square EMG exceeded 10 mV. MEPs were also rejected if peak-to-peak amplitude was less than 0.05 mV. This resulted in exclusion of 42 MEPs and an overall inclusion rate of 86% (258/300). A Shapiro-Wilk test showed that MEP amplitudes were significantly non-normal ($p < .05$); thus, a Wilcoxon Signed-Rank test was used to determine if there was significant MEP facilitation in paired pulse TMS trials compared to single pulse TMS trials across participants. Additionally, independent Student's t-tests were performed to compare MEP amplitudes between single and paired pulse trials within each participant in order to determine the proportion of participants who displayed significant ICF. Based on this analysis, premotor RT was then analyzed using 2 (ICF: ICF, non-ICF) x 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) mixed model ANOVA with repeated measures on Imperative stimulus and TMS condition. The presence of ICF was included as a factor to determine if there were any differences in the RT patterns observed between participants who exhibited ICF and those who did not, allowing an investigation of the possibility that the facilitatory effect of sub-threshold TMS on MEPs and RT has a similar underlying neural mechanism. Based on the finding in Experiment 1 that iEMG differences were only seen in the initial part of the EMG trace, iEMG for the first 100 ms ($iEMG_{100}$) of the ECR burst was calculated and analyzed using a 2 (Imperative stimulus: control, startle) x 3 (TMS condition: none, TMS, sham) RM ANOVA to determine if there were any differences in iEMG across conditions.

4. Experiment 3 Methods

4.1. Participants. 13 participants with normal or corrected to normal vision, no obvious upper body abnormalities, and no contraindications to TMS (Rossi et al., 2011) participated in the third experiment. Three participants did not exhibit consistent activation in SCM in response to a SAS, resulting in a final sample size of 10 participants (six female, four male; M_{age} = 25, SD = 7). All participants provided written informed consent before participating. The experiment was conducted in accordance with the ethical guidelines of the Health Sciences and Science Research Ethics Board at the University of Ottawa, and conformed to the latest revision of the Declaration of Helsinki.

4.2. Experimental Procedure. The experimental procedure in Experiment 3 was similar to that of Experiment 2 with modifications to the timing of TMS application in SAS trials and the form of sham TMS employed. As previously mentioned, the expected window for RT facilitation by sub-threshold TMS is from -20 to 20 ms with respect to the SAS. As such, in Experiment 3 TMS was applied simultaneous with the SAS (TMS_0) or 15 ms prior to the SAS (TMS_{-15}). While previous research has highlighted the importance of ensuring sham TMS is as similar as possible to real TMS (Duecker & Sack, 2015), methodological limitations prevent the ability to determine if TMS with a LM current direction over M1 results in ICF. Thus, in the present experiment TMS over vertex with a LM current direction was used as the sham condition, as neuroimaging studies have shown that TMS over vertex does not interfere with task-related activity within M1 (Jung, Bungert, Bowtell, & Jackson, 2016).

Similar to Experiment 2, two blocks of 15 MEPs (one in response to single pulse TMS and one in response to paired-pulse TMS) were collected prior to beginning the experiment. These were included to examine if RT facilitation and MEP facilitation by sub-threshold TMS

may involve a similar mechanism. Participants then completed 20^{o} targeted wrist extension movements in response to an auditory go-signal, in the same manner as Experiments 1 & 2. Following practice, experimental trials were performed as six blocks of 20 trials, for a total of 120 trials. Three of these blocks consisted of 11 control trials, three control trials with TMS, one SAS trial, two TMS_0 trials, two TMS_{-15} trials and one catch trial. The other three blocks consisted of 11 control trials, three control trials with sham TMS, one SAS trial, two $Sham_0$ trials, two $Sham_{-15}$ trials and one catch trial. Catch trials consisted of the presentation of the WS followed by a TMS pulse delivered after a variable time period. Participants were instructed to respond only to the go-signal, and that they were not supposed to respond on catch trials. These trials were included to control for the possibility that in the TMS_{-15} and $Sham_{-15}$ trials participants were responding to the TMS pulse, rather than to the SAS. Despite being told not to respond, participants responded on 83% (50/60) of trials; thus, RT in catch trials was also analyzed. The remainder of the experimental protocol (TMS, recording equipment and data reduction) were identical to those of Experiments 1 & 2. This resulted in the exclusion of 41 trials due to lack of an SCM burst on startle trials, 10 trials due to RTs above 300 ms, 40 trials due to RTs below 50 ms, 5 trials due to movement error and 6 trials due to the TMS not being delivered on TMS trials, and an overall inclusion rate of 92% (1098/1200). In addition, 43 MEPs from the ICF testing blocks performed prior to practice were discarded as 'bad MEPs' (see Experiment 2 methods for exclusion criteria), resulting in a total inclusion rate of 86% (257/300).

The statistical analyses performed for Experiment 3 were similar to those in Experiment 2 with a few modifications due to differences in the experimental protocol. To determine if the presentation of a SAS was leading to the involuntary release of the prepared response premotor RT, ECR1 burst duration, peak amplitude and $iEMG_{100}$ in control trials and startle trials (no

TMS) were analyzed using paired-samples t-tests. Premotor RT in startle conditions was then analyzed using a 2 (Subgroup: ICF, non-ICF) x 5 (SAS condition: SAS, TMS_0, TMS_{-15}, $Sham_0$, $sham_{-15}$) mixed model ANOVA with repeated measures on SAS condition to determine if there was an effect of TMS presentation on startle RT. For control trials, premotor RT was analyzed using a 2 (Subgroup: ICF, non-ICF) x 3 (TMS: none, sham, real) mixed model ANOVA with repeated measures on TMS to determine if there was an effect of TMS on control RT. As participants responded on 83% of catch trials, premotor RT on these trials was also analyzed; however, due to the small number of trials for each participant these trials were combined across participants for analysis. A Shapiro-Wilk test showed that RT in catch trials was significantly non-normal ($p < .05$); thus, a Mann-Whitney U test was used to determine if there was a difference in RT in response to real and sham TMS. A Mann-Whitney U test was used as there were limited catch trials, and not all participants responded on all catch trials, preventing the ability to perform paired-samples analyses. To determine the effect of sub-threshold TMS on movement kinematics in startle conditions ECR1 burst duration, peak amplitude and $iEMG_{100}$ were analyzed using separate one-way (SAS condition: SAS, TMS_0, TMS_{-15}, $Sham_0$, $Sham_{-15}$) RM ANOVAs. Similarly, ECR1 burst duration, peak amplitude and $iEMG_{100}$ in control conditions were analyzed using separate one-way (TMS: none, sham, real) RM ANOVAs to determine the effect of TMS on movement kinematics in control trials.

5. Experiment 1 Results

5.1. Premotor RT. Premotor RT values across all six conditions are shown in Figure 1. Analysis revealed a significant main effect of Stimulus on premotor RT, $F(1, 13) = 50.953$, $p < .001$, $\eta^2_p = .797$, as well as a significant main effect of TMS, $F(1.43, 26) = 27.244$, $p < .001$, $\eta^2_p = .677$. However, these effects were superseded by a significant interaction between the factors,

$F(2, 26) = 23.084$, $p < .001$, $\eta^2_p = .640$. Post-hoc tests using Tukey's HSD indicated that in trials with the control (82 dB) IS, sham TMS ("click") resulted in significantly faster premotor RTs than trials where no additional stimulus was presented ($p < .05$). Furthermore, control IS trials with real TMS led to significantly faster RTs than both trials with no TMS and sham TMS ($p < .05$). In contrast, there were no significant differences in premotor RT between any of the TMS conditions when a SAS was presented ($p > .05$).

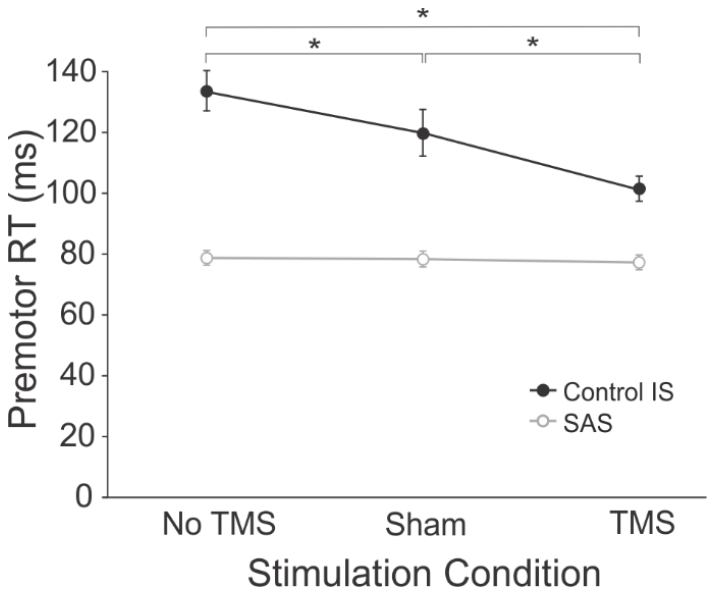

Figure 1. Mean (SE) premotor reaction time (RT) observed across TMS and acoustic stimulation conditions in Experiment 1 (n=14). Premotor RT in the no TMS (None), Sham TMS, and real TMS conditions are shown as a function of acoustic stimulus condition (Control IS vs SAS). Asterisks (*) denote significant ($p < .05$) differences between TMS conditions in response to the control IS, as there were no significant differences between the SAS conditions.

5.2. SCM activity. Analysis revealed that there were no significant differences in the proportion of trials where a startle response was elicited ($p > .05$). The percentage of SAS trials where a startle response was elicited, measured by the presence of an SCM burst, was 94% (SD = 19), 88% (SD = 24), and 96% (SD = 8) in the no TMS, sham TMS, and real TMS conditions, respectively.

5.3. EMG activity. Ensemble averaged EMG across all participants is shown for all TMS conditions in Figure 2 with control (non-SAS) trials in Figure 2a, and SAS trials in Figure 2b. Analysis of the duration of the initial agonist burst (ECR1) indicated that there were no significant differences in duration due to Stimulus or TMS ($p > .05$). For peak amplitude in ECR1, a main effect of Stimulus was found, $F(1, 13) = 11.393$, $p < .001$, $\eta^2_p = .467$, indicating that the peak EMG amplitude for the ECR1 burst was significantly greater in SAS (0.35 mV, SD = 0.18) than control IS (0.30 mV, SD = 0.16) conditions. There were no significant differences in ECR1 peak amplitude in the different TMS conditions, and no interaction between the factors ($p > .05$). Analysis of the iEMG across the entire movement trace revealed a significant main effect of Time, $F(5,1) = 17.608$, $p > .001$, $\eta^2_p = .575$, as well as a significant main effect of Imperative stimulus, $F(1,13) = 14.406$, $p = .002$, $\eta^2_p = .526$. However, these effects were superseded by a significant interaction between the factors, $F(5,65) = 2.455$, $p = .042$, $\eta^2_p = .159$. Post-hoc analyses indicated that in control trials iEMG was significantly greater at Time 2 than at Times 1, 4, 5 and 6. In addition, iEMG was significantly greater at Times 3 and 4 than at Time 6. In contrast, iEMG in startle trials was significantly smaller at Time 6 than at all other time points, and iEMG was significantly greater at Time 2 than at all other time points. There was no main effect of TMS or any interactions between TMS condition and any other factors (all p-values > .05). Taken together, these results suggest there was no effect of TMS on the movement pattern

elicited in either the startle or control conditions; however, in startle conditions the ECR1 burst was significantly larger than in the control conditions.

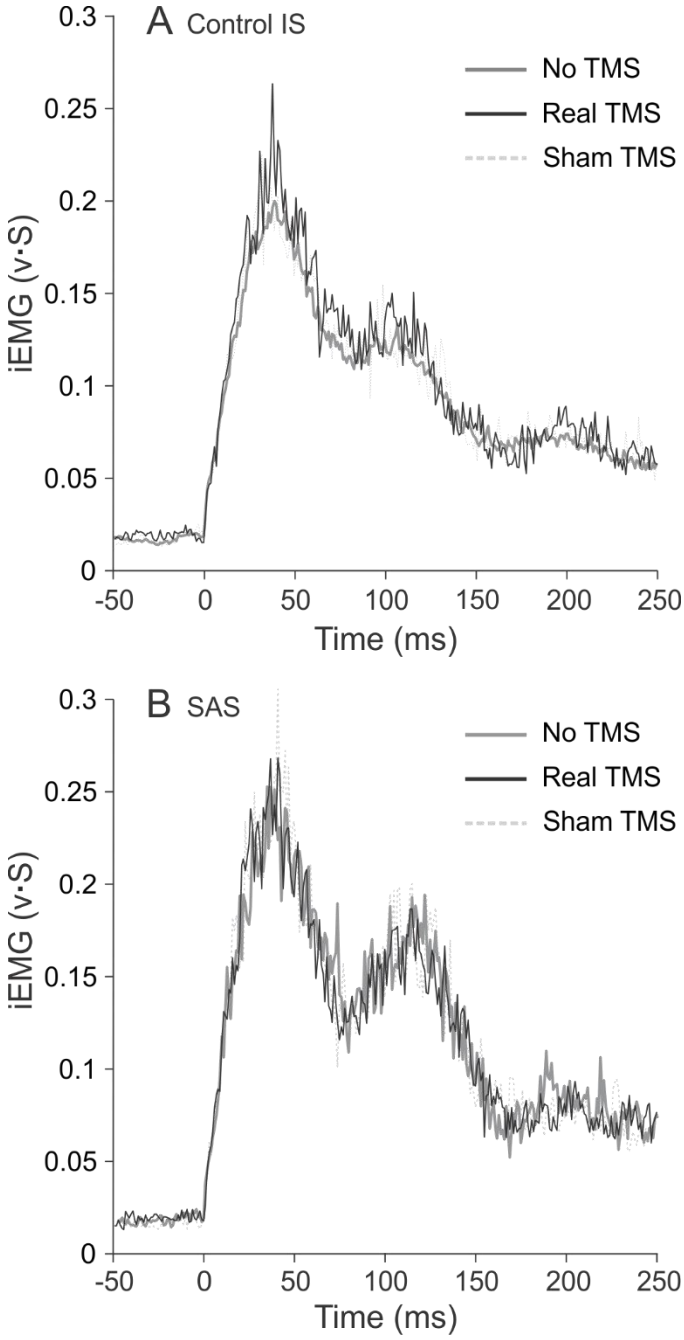

Figure 2. Ensemble averaged EMG traces across all participants in Experiment 1 (n=14) for each TMS stimulation condition in both the control (A), and SAS (B) imperative stimulus conditions. Note: All individual traces were time-locked to EMG onset in each given trial.

5.4. TMS. The mean resting motor threshold of participants was 40% (SD = 8) of maximal stimulator output, resulting in a mean TMS intensity of 32% (SD = 6) of maximal stimulator output being used during the experiment.

6. Experiment 2 Results

6.1. ICF. Analysis of the peak-to-peak amplitude of baseline MEPs (Median = 0.259) and ICF MEPs (Median = 0.269) revealed no significant differences between conditions, $T = 26$, $p > .05$, $r = -.048$. However, analysis of MEP amplitude within each participant using individual student's t-tests indicated that the conditioning stimulus led to significantly larger test MEPs in 5/10 participants. As such, data from these participants were separated for further analysis of Premotor RT.

6.2. Premotor RT. Analysis of premotor RT when separated by ICF subgroup revealed no significant main effect of group and no interactions with group (all p-values > .05), indicating that any effects of imperative stimulus and TMS on RT were not differentiated based on the presence or absence of a significant ICF effect. As such, figure 3 shows the premotor RT values for all six conditions of all participants as a whole. Analysis did reveal a main effect of Stimulus, $F(1,8) = 82.159$, $p < .001$, $\eta^2_p = .911$, as well as a main effect of TMS, $F(2,16) = 14.275$, $p < .001$, $\eta^2_p = .641$; however, these were superseded by a significant interaction between the Stimulus and TMS, $F(2,16) = 16.021$, $p < .001$, $\eta^2_p = .667$. Post-hoc analysis using Tukey's HSD showed that in control IS trials sham TMS led to significant reductions in premotor RT ($p < .05$), and real TMS led to significantly faster RTs than both no TMS and sham TMS ($p < .05$), whereas in SAS trials there were no significant differences in RT between trials with no TMS, sham TMS, or real TMS ($p > .05$).

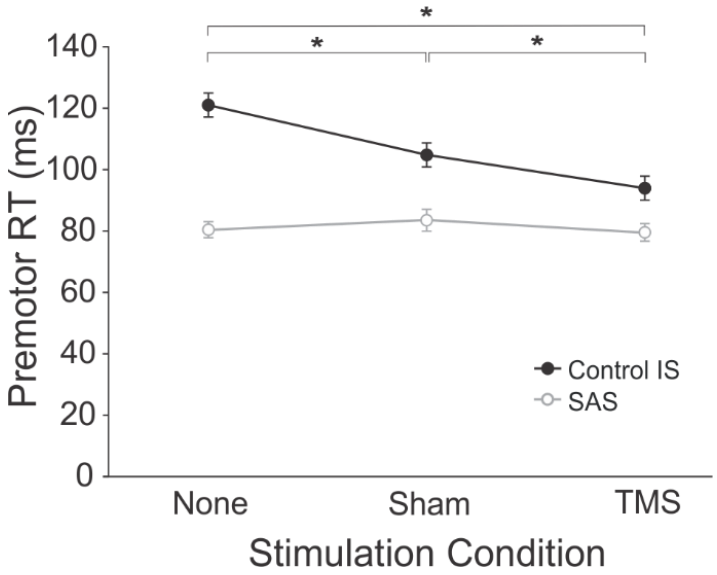

Figure 3. Mean (SE) premotor reaction time (RT) observed across TMS and acoustic stimulation conditions in Experiment 2 (n=10). Premotor RT in the no TMS (None), sham TMS, and real TMS conditions are shown as a function of acoustic stimulus condition (Control IS vs SAS). Asterisks (*) denote significant ($p < .05$) differences between TMS conditions in response to the control IS, as there were no significant differences between the SAS conditions.

6.3. SCM activity. Across the SAS conditions a startle response was elicited on 97% (SD = 6) of trials with no TMS, 100% of trials with sham TMS, and on 98% (SD = 8) of trials with real TMS. Analysis of these values indicated that there were no significant differences between the proportion of startle responses elicited between the three SAS conditions ($p > .05$).

6.4. EMG activity. Analysis revealed a significant main effect of Stimulus on the duration of the ECR1 burst, $F(1, 9) = 6.038$, $p = .036$, $\eta^2_p = .402$. This result indicated that the duration of the ECR1 burst was significantly shorter in startle trials (74.0 ms, SD = 13) compared to control trials (78.0 ms, SD = 12). There was no significant effect of TMS on ECR1 burst duration and no interaction between the factors ($p > .05$). Similarly, there was a significant main effect of Stimulus on ECR1 peak amplitude, $F(1, 9) = 15.583$, $p = .003$, $\eta^2_p = .634$. This main effect was due to significantly greater peak amplitude in startle (0.56 mV, SD = 0.32) than control (0.50, SD = 0.29) conditions. Analysis of the $iEMG_{100}$ of ECR1 burst also showed similar results, with a main effect of stimulus, $F(1, 9) = 11.00$, $p = .009$, $\eta^2_p = .550$, indicating that the $iEMG_{100}$ in SAS trials (.026 mV ·s, SD = .016) was significantly greater than in control trials (0.023 mV·s, SD = .016).

6.5. TMS. The mean resting motor threshold in participants was 41% (SD = 6) of maximal stimulator output. This resulted in a mean TMS intensity of 33% (SD = 5) of maximal stimulator output being used for the experimental protocol, and an average intensity of 50 % (SD = 7) of maximal stimulator output being used for the test pulse in the MEP testing blocks.

7. Experiment 3 Results

7.1. Confirmation of StartReact effect. Analysis of control and startle trials with no TMS revealed that premotor RT was significantly slower in control trials (129.6 ms, SD = 18) than in startle trials (84.6 ms, SD = 13). In contrast, there were no significant differences between these

conditions for ECR1 burst duration, peak amplitude or $iEMG_{100}$ ($p > .05$), indicating that presentation of the SAS resulted in the involuntary triggering of the prepared movement.

7.2. ICF. Analysis of the peak-to-peak amplitude of unconditioned MEPs (Median = 0.222) and conditioned MEPs (Median = .214) revealed no significant differences between these conditions, $T = 11$, $p > .05$, $r = -.532$. However, individual student's t-tests performed for each participant indicated that MEP amplitude was significantly greater in response to paired-pulse TMS than single pulse TMS in 5/10 participants. To determine if the facilitatory effect of sub-threshold TMS on MEP amplitude and RT involve a similar mechanism, participants were divided into two subgroups (ICF and non-ICF) for premotor RT analysis.

7.3. Premotor RT. Figure 4a shows the premotor RT values for all three control imperative stimulus conditions. Analysis revealed a significant main effect of condition, $F(2,18) = 21.537$, $p < .001$, $\eta^2_p = .705$, and post-hoc analysis indicated that RT was significantly faster in the control TMS and control sham conditions, with no significant differences between these conditions ($p > .05$). There was no significant difference between the ICF and non-ICF groups, as well as no interaction involving subgroup (all p values > .05). Figure 4b shows the premotor RT values for all five startle conditions; analysis revealed a significant main effect of condition, $F(4,36) = 11.77$, $p < .001$, $\eta^2_p = .567$. Post-hoc analysis using Tukey's HSD indicated that RT in the SAS_{-15} and SAS_{-15} sham conditions was significantly faster than in all other conditions, with no difference between any of the other conditions. There were no main effects or interactions involving sub-group (all p-values > .05), indicating that in both control and startle trials the effects of TMS on premotor RT were similar in those who exhibited ICF and those who did not. Analysis of catch trials showed a trend towards RT being faster in response to real TMS (100.0

ms, SD = 43) than to sham TMS (129.5 ms, SD = 63); however, this effect did not reach significance ($p = .085$).

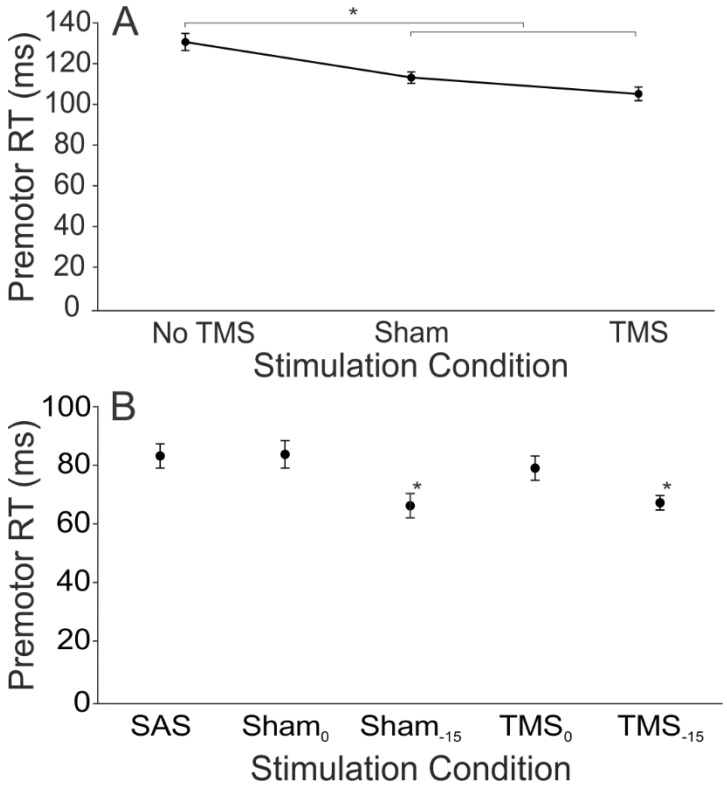

Figure 4. Mean (SE) premotor reaction time (RT) across TMS stimulation condition for the control (A) and startle (B) imperative stimulus conditions in Experiment 3 (n=10). Asterisks (*) denote significant ($p > .05$) differences between TMS conditions. Note: premotor RT was significantly faster in startle trials than in non-startle trials.

7.4. SCM activity. Across SAS conditions a startle response was elicited in 79% (SD = 26) of trials with no TMS, 90% (SD = 14) of trials in the TMS_0 condition, 88% (SD = 18) of trials in the TMS_{-15} condition, 87% (SD = 17) of trials in the TMS_0 condition, and 87% (SD = 25) of trials in the TMS_{-15} condition. Analysis revealed that there were no significant differences between the proportion of startle responses elicited between SAS conditions ($p > .05$).

7.5. EMG activity. Analysis of SAS trials revealed no significant differences between conditions for ECR1 burst duration, peak amplitude or $iEMG_{100}$ ($p > .05$). Similarly, there were no significant differences between TMS conditions in non-startle trials with respect to ECR1 burst duration, peak amplitude or $iEMG_{100}$ ($p > .05$).

7.6. TMS. The mean resting motor threshold for participants was 43% (SD = 9) of maximal stimulator output. This resulted in a mean intensity of 34% of maximum stimulator output being used during experimental trials, and a mean intensity of 52% of maximum stimulator output being used for the test pulse in the MEP testing blocks.

8. General Discussion

The purpose of the present experiments was to determine if application of sub-threshold TMS applied over M1 following a SAS would lead to facilitation of startle elicited RT, providing evidence that the cortex plays a role in startle-triggered reduction of RT. While the StartReact effect has been extensively studied (Carlsen, Maslovat, et al., 2012; Valls-Solé et al., 2008), the neural mechanism underlying this phenomenon remains unclear. Specifically, previous work has provided conflicting evidence regarding cortical involvement in startle-facilitated RTs (Alibiglou & MacKinnon, 2012; Honeycutt & Perreault, 2012; Nonnekes et al., 2014; Stevenson et al., 2014), which has led to two primary competing hypotheses: 1) a subcortical storage hypothesis

(Carlsen et al., 2004a; Nonnekes et al., 2014; Valls-Solé et al., 1999) and 2) a cortical storage hypothesis (Alibiglou & MacKinnon, 2012; Carlsen, Maslovat, et al., 2012). The primary result from the present study is that sub-threshold TMS applied over M1 shortly following the go-signal facilitated RT in control trials compared to both no TMS and sham TMS; however, sub-threshold TMS did not have a similar effect on RT following startle. This finding was consistent regardless of the timing of TMS application in startle trials. As such, these results suggest that M1 may in fact play a limited role in the pathway of the StartReact effect, providing added support for the subcortical storage hypothesis.

In all three experiments a SAS elicited consistent SCM activation in all retained participants, indicating that a startle reflex was successfully evoked. Furthermore, although premotor RT was strongly facilitated by the SAS, similar movement-related EMG patterns were observed in control and startle trials. EMG amplitude was significantly greater for the initial agonist burst in startle trials in Experiments 1 & 2, which is occasionally seen in response to a SAS, and is believed to be due to a larger initiation signal being elicited by the SAS, or to summation of the startle volley and voluntary command (Carlsen, Almeida, & Franks, 2012; Carlsen et al., 2013; Siegmund et al., 2001). Initial agonist burst was also significantly shorter in startle trials than control trials in Experiment 2; however, this was not seen in Experiments 1 & 3, and as such likely represents a chance occurrence. These results indicate that presentation of a SAS led to the early and involuntary triggering of the planned movement (Carlsen et al., 2004b), allowing for investigation of the underlying neural mechanism. Previously, the use of relatively high intensity (160-180% of rMT) supra-threshold TMS during a RT task showed that inducing a cortical silent period in M1 not only delayed normal voluntary RT, but also delayed startle-elicited RTs to a similar extent. This was used as evidence that M1 is involved in the neural

pathway underlying the StartReact effect (Alibiglou & MacKinnon, 2012; Stevenson et al., 2014). A limitation of employing supra-threshold TMS to investigate cortical involvement is that it can also lead to descending activity in the cortico-reticular pathway (Fisher et al., 2012; Nonnekes, Carpenter, Inglis, Duysens, & Weerdesteyn, 2015), putting the locus of the effects of supra-threshold TMS on the StartReact effect into question. The present study applied *sub-threshold* TMS in a startle paradigm to address this limitation, as sub-threshold TMS does not lead to any residual descending activity in the spinal cord (Di Lazzaro et al., 2012). It was hypothesized that if the cortex was involved in the StartReact effect, startle elicited RT would be facilitated in the same manner as control RT. However, the results from SAS trials in Experiment 1 showed no significant differences in startle trial RT in response to either real or sham TMS. This is in contrast to control IS trials where premotor RT was significantly facilitated by the application of TMS (Figure 1). These results appear to support the subcortical storage hypothesis; however, in Experiment 1 the sham condition did not replicate the full sensory experience of true TMS, and TMS was applied with different timing between control and SAS trials, which may have contributed to some of the observed differences, limiting the ability to elucidate whether or not the cortex has a role in startle elicited RT.

Previous research has shown that both the air and bone-conducted auditory aspects of a TMS click must be taken into account when considering sham TMS stimulation conditions (Nikouline, Ruohonen, & Ilmoniemi, 1999). Furthermore, many commonly used sham conditions do not fully replicate the sensory experience (i.e. the auditory click and vibration of the scalp) associated with receiving a TMS pulse, which must be controlled for to ensure experimental results are in fact due to the specific neural effects of TMS (Duecker & Sack, 2015). Based on these observations, the sham condition used in Experiment 1 may not have been

sufficient to determine if the RT facilitation seen could be attributed to a direct effect of sub-threshold TMS on M1. As such, the sham condition used in Experiment 2 employed real TMS applied over M1 (using the same intensity as that in the real TMS condition), but with a coil orientation eliciting a LM current direction rather than a PA current direction. This sham condition replicated the entirety of the sensory experience of TMS, but because a LM current direction activates different cortical neurons than a PA current direction (Di Lazzaro et al., 2012), it has been shown to not result in similar cortical effects (e.g., ICF; Ziemann et al., 1996). Similar to Experiment 1, the control trial results of Experiment 2 showed that sham TMS resulted in significant facilitation of RT, but importantly, real TMS significantly reduced RT in comparison to both no TMS and sham TMS (Figure 3). This result provides strong evidence that sub-threshold TMS had a direct effect on the cortex that caused reductions in RT beyond those resulting from speeded sensory effects. These results also indicate that in certain situations TMS with an altered current direction can be used to address the limitations found in many sham and control TMS conditions (Duecker & Sack, 2015). Specifically, real TMS with an altered current direction fully replicates the sensory experience of TMS on the same location of the scalp, and it also had altered effects on neural processes and behavior in a RT paradigm.

A second possible confounding factor in Experiment 1 was the timing of TMS application; TMS was applied 15 ms following the SAS in startle trials and 30 ms following the go-signal in control trials. This timing was chosen based on a neural accumulator model, which indicates that initiation-related activation processes begin approximately 15 ms earlier when the response is elicited by startle compared to control trials (Maslovat, Carter, et al., 2014). However, it is possible that this difference in timing of TMS application may have been responsible for the differences in RT patterns between the control and startle conditions in

Experiment 1. To address this possibility, in Experiment 2 TMS was applied at the same time point (30 ms following the control go-signal or SAS) in both control and startle trials. The pattern of results in Experiment 2 was similar to that of Experiment 1; that is, there was no significant effect of real or sham TMS on startle trial RT even though RT was significantly facilitated when TMS was applied after the control go-signal. These results further support a model whereby sufficient details of the intended response are planned and stored subcortically, possibly in brainstem structures, and suggest that the cortex may have only a limited role in the initial output of responses that have been facilitated by startle.

The altered timing of TMS application in Experiment 2 was used to control for the possibility that TMS was applied too early in SAS trials in Experiment 1; however, it is also possible that TMS was delivered too late to have an effect on startle RT. The effect of sub-threshold TMS on control RT has been extensively studied, with results indicating that RT facilitation occurs when TMS is applied approximately 90-150 ms prior to average RT (Hashimoto et al., 2004; Pascual-Leone, Valls-Sole, Brasil-Neto, Cohen, & Hallett, 1994; Terao et al., 1997). Due to the increased rate of accumulation of initiation-related activation in startle conditions (Maslovat, Carter, et al., 2014), the expected window for RT facilitation becomes 60 – 100 ms prior to average startle RT. As startle RT in Experiments 1 & 2 was approximately 80 ms, this suggests TMS facilitation could be expected to occur up to 20 ms prior to presentation of the SAS. Based on this, TMS in startle trials was applied with the SAS and 15 ms prior to the SAS in Experiment 3. The results of control trials showed that premotor RT in both sham and real TMS conditions was significantly faster than in the no TMS condition (Figure 4a). In contrast to Experiments 1 &2, real TMS did not result in significantly faster RT than sham TMS; however, there was a trend towards RT being faster in the real TMS condition. In addition,

despite participants being told not to respond on catch trials, a trend towards RT being faster on catch trials in response to real TMS than to sham TMS was also seen, suggesting that TMS also reduced RT partly through a direct effect on the cortex in Experiment 3. The results of startle trials showed that RT was significantly faster in both conditions where TMS was applied 15 ms prior to the SAS, with no differences between any of the other groups (Figure 4b). These results appear to suggest that startle RT can be facilitated by the application of sub-threshold TMS, providing evidence for cortical involvement in the StartReact effect; however, participants responded on 83% of catch trials, and the RT reductions seen were approximately 15 ms. In addition, studies presenting a SAS following the go-signal have shown that voluntary and startle-related initiation activation jointly contribute to the significant reductions in RT seen under these conditions (Maslovat, Carter, et al., 2014; Maslovat, Drummond, Carter, & Carlsen, 2015). Combined, these results suggest that when TMS was presented prior to the SAS participants were initially voluntarily responding to the TMS pulse, with involuntary startle-related initiation activation beginning after SAS presentation, resulting in reduced RT. As such, the results of Experiment 3 provide further support for a model where a SAS involuntarily triggers the release of a sub-cortically stored movement, with little or no involvement of the cortex.

While the primary purpose of these experiments was to investigate the role of the cortex in startle-elicited RT, the results from the control IS condition also provide insight into the mechanisms responsible for the RT facilitation induced by sub-threshold TMS applied early in the RT interval. Experiments where sham TMS resulted in similar effects on RT as real TMS has led some researchers to suggest that sub-threshold TMS reduces RT solely through intersensory facilitation and stimulus intensity effects (Hashimoto et al., 2004; Terao et al., 1997). In contrast, Soto et al. (2010) showed that the facilitatory effects of sub-threshold TMS were similar in both

simple and choice RT paradigms, suggesting that TMS leads to RT facilitation by enhancement of motor processes. The results of Experiment 1 showed that sham TMS in the form of an auditory click presented early in the RT interval resulted in significantly reduced RT, providing evidence that the non-specific auditory aspect of TMS partially reduced RT through stimulus intensity effects. However, real TMS resulted in further RT speeding compared to sham TMS, indicating that sub-threshold TMS also affected motor processes in cortex that led to further reductions in RT. The results of Experiments 2 & 3 where sham TMS conditions that fully replicated the sensory experience of real TMS were employed displayed similar RT patterns. As such, the TMS-facilitated reductions in RT can be attributed to an additive effect of both speeded sensory processes, as well as a direct facilitatory effect of TMS on the cortex.

Despite the direct facilitatory effect of TMS on the cortex seen in control trials there was no effect in startle trials, suggesting that the cortex does not play a role in the neural pathway underlying the StartReact effect. However, it is possible that the effects of TMS may only be seen later in the movement in startle trials due to the significantly reduced RT latency in comparison to control trials. To address this possibility, iEMG was analyzed throughout the entire movement in Experiment 1, and it was found that while the EMG burst was greater in startle trials than control trials early in the movement, TMS did not have a significant effect on the EMG profile at any time point in either control or startle trials (see Figure 2). These results indicate that not only did TMS not have an effect on RT in startle trials, TMS also did not have an effect on startle-elicited movements later on in the movement. Similar results were seen in control trials by Hashimoto et al. (2004), who applied sub-threshold TMS at varying times following the go-signal and compared the integrated EMG across conditions, with results showing no effects of TMS. The authors suggested that sub-threshold TMS solely temporally

facilitates neuronal processes in M1 without affecting the centrally stored motor program. These results align with Pascual-Leone et al.'s (1992) proposal that sub-threshold TMS facilitates the transfer of a stored motor program to the neurons responsible for execution of the movement. As such, while the results of the iEMG analysis cannot provide evidence for cortical involvement in the StartReact, they do support a possible mechanism underlying the facilitatory effect of sub-threshold TMS on RT.

Considering this mechanism of RT facilitation within an ICF paradigm, sub-threshold TMS applied during the RT interval may add extra energy to the motor pathway, increasing cortical excitability and resulting in RT facilitation in the same manner it leads to MEP facilitation. The results of Experiments 2 & 3, where the degree of ICF was explicitly tested prior to beginning testing, do not appear to support this hypothesis. In all three experiments the application of sub-threshold TMS led to significantly reduced RT in response to the control IS; however, in both Experiments 2 & 3 only 50% of participants exhibited conditioned MEPs that were significantly larger than control MEPs. When the presence of ICF was included as a factor in analysis of the RT data, results of both experiments showed no significant difference in RT patterns between those who exhibited ICF and those who did not. These results suggest that the facilitatory effects of sub-threshold TMS on RT and on MEPs likely do not share the same underlying neural mechanism.

In conclusion, the results of the current study showed that sub-threshold TMS applied over M1 facilitated control RT through both speeded sensory effects as well as addition of energy to M1. It appears that this direct effect of TMS on the cortex exclusively facilitates temporal processes occurring in M1, while leaving the central motor program unaltered. In contrast to the results from control trials, sub-threshold TMS did not lead to changes in startle

elicited RT, whether it was applied prior to, concurrent with, or following the SAS. The results from the startle condition can be interpreted as evidence that a SAS results in the early triggering of a sub-cortically stored motor program, with limited to no involvement of the cortex. Alternatively, because startle elicited RTs were similar across conditions in all three experiments, the use of a simple ballistic wrist extension movement may have resulted in a RT floor effect whereby humans are not capable of responding any faster, even if the response was cortically mediated. More complex movements are known to have slower RTs in both control (Klapp, 2003) and startle conditions (Maslovat, Klapp, Jagacinski, & Franks, 2014); thus, future research should employ a more complex movement within the present paradigm to better elucidate the involvement of the cortex in the neural mechanism underlying startle-facilitated RTs.

Disclosures.

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Appendix C: Graphic representation of hypothesis

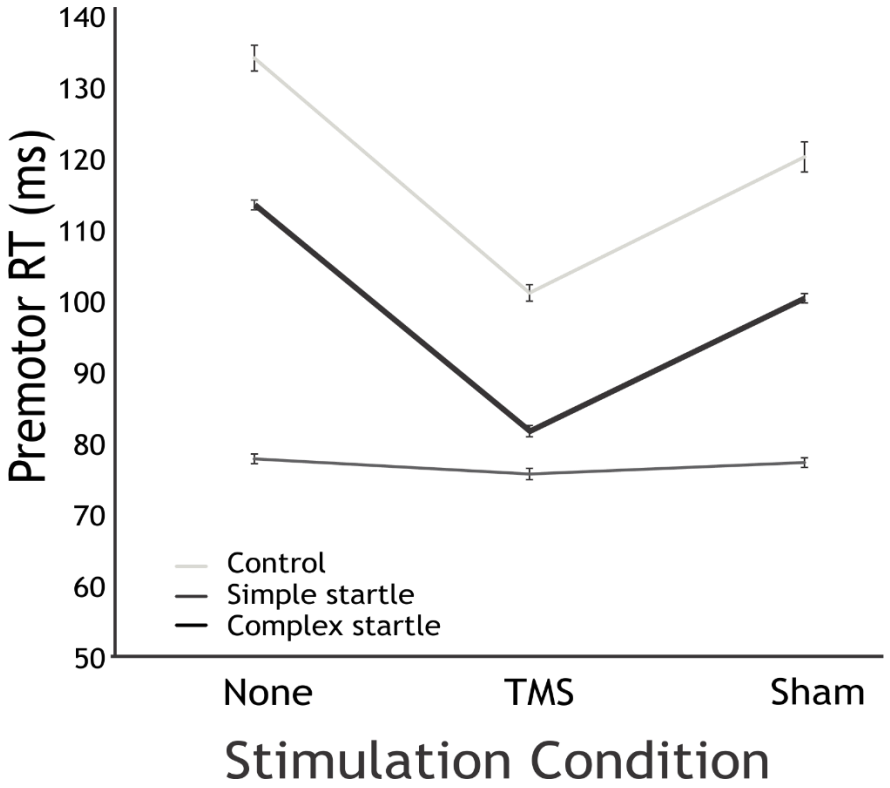

Figure A-2. Graph depicting the hypothesis for the present experiment. Using a complex movement is expected to increase startle RT, allowing room for RT facilitation of sub-threshold TMS in startle trials.

Appendix D: Schematic of the timeline of TMS application relative to neural processing

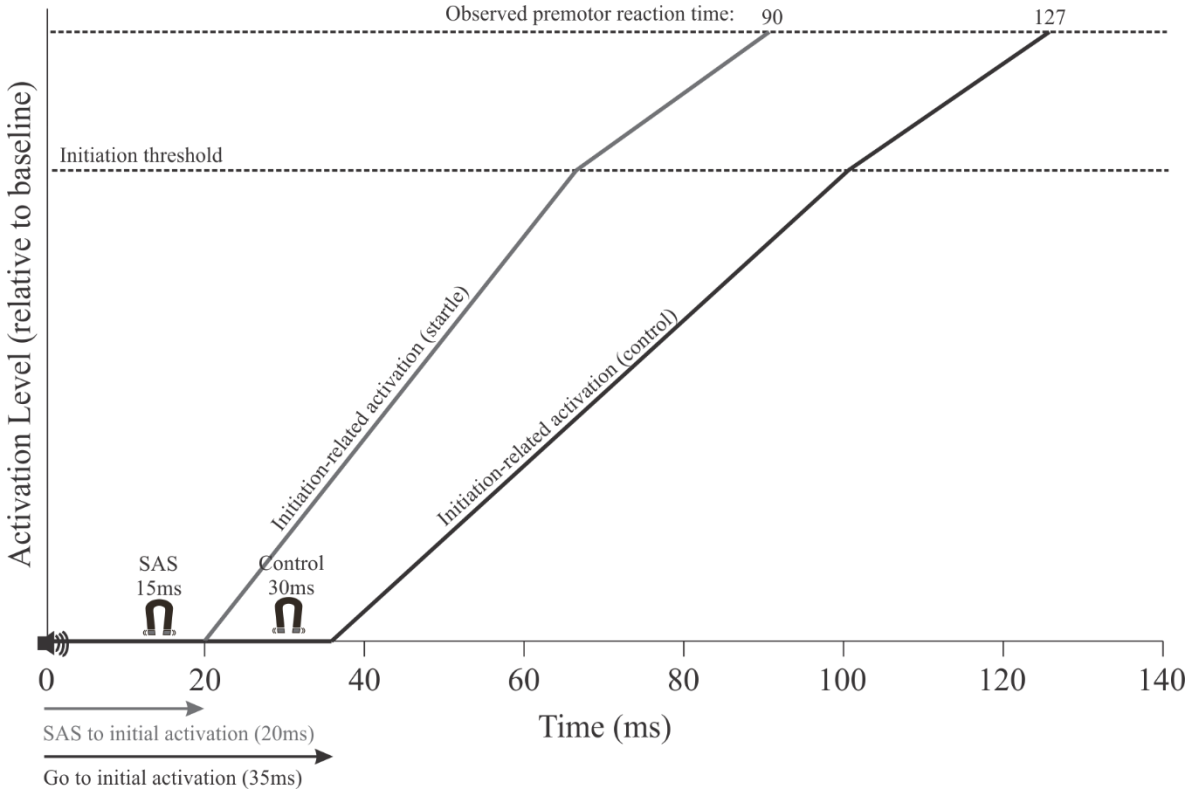

Figure A-3. Timing of TMS application relative to initiation-related activation according to the neural accumulator model. It has been shown that initiation-related activation begins earlier in startle trials than in control trials (Maslovat et al., 2014). Based on this, TMS in the present experiment was applied just prior to the expected increases in activation in control and startle trials.

Appendix E: TMS safety questionnaire

5/16/2013

Safety Screening Questionnaire for Transcranial Magnetic Stimulation

Please answer the following questions by putting a check mark (√) in the appropriate YES or NO box.

1. Have you ever had an adverse reaction to transcranial magnetic stimulation?	YES ☐	NO ☐
2. Had a seizure?	YES ☐	NO ☐
3. Had an EEG (electroencephalogram)?	YES ☐	NO ☐
4. Had a stroke?	YES ☐	NO ☐
5. Had a head injury (include neurosurgery)?	YES ☐	NO ☐
6. Do you have any metal in your head (outside the mouth) such as shrapnel, surgical clips, or fragments from welding or metalwork?	YES ☐	NO ☐
7. Do you have any implanted devices such as cardiac pacemakers, medical pumps, or intracardiac lines?	YES ☐	NO ☐
8. Do you suffer from frequent or severe headaches?	YES ☐	NO ☐
9. Have you ever had any other brain-related condition?	YES ☐	NO ☐
10. Have you ever had illness that caused brain injury?	YES ☐	NO ☐
11. Are you taking any medications?	YES ☐	NO ☐
12. Does anyone in your family have epilepsy?	YES ☐	NO ☐
13. Are you pregnant?	YES ☐	NO ☐

PARTICIPANT NAME: _____

PARTICIPANT SIGNATURE: _____ DATE: _____
OR
SIGNATURE OF LEGALLY
AUTHORIZED INDIVIDUAL

Appendix F: Ethics certificate

File Number: H03-12-03 **Date (mm/dd/yyyy):** 03/27/2015

Université d'Ottawa University of Ottawa
Bureau d'éthique et d'intégrité de la recherche Office of Research Ethics and Integrity

Ethics Approval Notice

Health Sciences and Science REB

Principal Investigator / Supervisor / Co-investigator(s) / Student(s)

First Name	Last Name	Affiliation	Role
Anthony	Carlsen	Health Sciences / Human Kinetics	Principal Investigator
Erin K.	Cressman	Health Sciences / Human Kinetics	Co-investigator
Neil	Drummond	Health Sciences / Human Kinetics	Co-investigator
Julie	Nantel	Health Sciences / Human Kinetics	Co-investigator
Michael	Carter	Health Sciences / Human Kinetics	Research Assistant
Amanda	Chiucchi	Health Sciences / Human Kinetics	Research Assistant
Joelle	El-Hajj	Health Sciences / Human Kinetics	Research Assistant
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Victoria	Smith	Health Sciences / Human Kinetics	Research Assistant

File Number: H03-12-03

Type of Project: Professor

Title: Investigating How Modulating Cortical Excitability Affects Motor Performance

Renewal Date (mm/dd/yyyy)	Expiry Date (mm/dd/yyyy)	Approval Type
04/05/2015	04/04/2016	Ia

(Ia: Approval, Ib: Approval for initial stage only)

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File Number: H03-12-03 **Date (mm/dd/yyyy)**: 03/27/2015

Université d'Ottawa University of Ottawa
Bureau d'éthique et d'intégrité de la recherche Office of Research Ethics and Integrity

Special Conditions / Comments:
N/A

This is to confirm that the University of Ottawa Research Ethics Board identified above, which operates in accordance with the Tri-Council Policy Statement (2010) and other applicable laws and regulations in Ontario, has examined and approved the ethics application for the above named research project. Ethics approval is valid for the period indicated above and subject to the conditions listed in the section entitled "Special Conditions / Comments".

During the course of the project, the protocol may not be modified without prior written approval from the REB except when necessary to remove participants from immediate endangerment or when the modification(s) pertain to only administrative or logistical components of the project (e.g., change of telephone number). Investigators must also promptly alert the REB of any changes which increase the risk to participant(s), any changes which considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project and safety of the participant(s). Modifications to the project, including consent and recruitment documentation, should be submitted to the Ethics Office for approval using the "Modification to research project" form available at: http://research.uottawa.ca/ethics/submissions-and-reviews.

Please submit an annual report to the Ethics Office four weeks before the above-referenced expiry date to request a renewal of this ethics approval. To close the file, a final report must be submitted. These documents can be found at: http://research.uottawa.ca/ethics/submissions-and-reviews.

If you have any questions, please do not hesitate to contact the Ethics Office at extension 5387 or by e-mail at: ethics@uOttawa.ca.

Signature:

Mélanie Rioux
Ethics Coordinator
For Catherine Paquet, Director of the Office of Research Ethics and Integrity

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