Characteristics of preceding Ia activity on post-activation depression in health and disease

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Abstract

Previous activation of the soleus Ia afferents causes a depression in the amplitude of the H-reflex. This mechanism is referred to as post activation depression (PAD) and is suggested to be presynaptically mediated. Using a paired reflex depression paradigm (eliciting two H-reflexes with conditioning-test intervals from 80 ms to 300 ms), PAD was examined in a group of healthy individuals and a group of hemiplegic patients. Healthy individuals showed substantial depression of the test H-reflex at all intervals. While the patient group showed substantially less depression at all intervals, increasing the interval between the two reflexes sharply reduced the depression. In a separate experiment, we varied the size of the conditioning H-reflex against a constant test H-reflex. In healthy individuals, by increasing the size of the conditioning H-reflex, the amplitude of the test H-reflex exponentially decreased. In the patient group, however, this pattern was dependent on the conditioning-test interval; increasing the size of the conditioning H-reflex caused an exponential decrease in the size of the test reflex at intervals shorter than 150 ms. This pattern was similar to that of healthy individuals. However, conducting the same protocol at a longer interval (300 ms) in these patients resulted in an abnormal pattern (instead of an exponential decrease in the size of the test reflex exaggerated responses were observed). Fisher discriminant analysis suggested that these two patterns (which differed only in the timing between the two stimuli) were substantially different from each other. Therefore, it is suggested that the abnormal pattern of PAD in hemiplegic stroke patients could be a contributing factor for the pathophysiology of spasticity.
Introduction

Depression of transmission between Ia fibers and alpha motoneurons due to the history of activation of the fibers is a spinal inhibitory mechanism which occurs after a passive stretch of the agonist muscle, tapping its tendon or electrically stimulating the afferents repetitively or at a low frequency (Crone and Nielsen 1989). The term post activation depression (PAD) or homosynaptic depression has been used to indicate that the same afferents are involved in this depression (Crone and Nielsen 1989). Modulation of this mechanisms during functional tasks such as muscle activation and movement initiation (Stein et al. 2007) has been reported but its functional role in shaping human movement is not very well understood (Knikou 2008).

In noninvasive experimental settings, this inhibitory mechanism can be quantitatively evaluated by different H-reflex protocols. One method is to elicit single pulses of nerve stimulation at different frequencies and measure the amplitude of the first and last H-reflex. Another method is to elicit two H-reflexes with equal stimulus intensities at a constant frequency but varying the interstimulus delay. The protocol is referred to as paired reflex depression (PRD). In this protocol, the first H-reflex is regarded as the conditioning stimulus and the second H-reflex is regarded as the test (conditioned) reflex. Studies over the past few decades have shed considerable light on the mechanism of this inhibition. It is widely agreed that the mechanism is confined to the presynaptic terminals of the Ia fibers and does not depend on alpha motoneurons excitability. Furthermore, PAD does not interfere with heteronymous sources to the motor pool (Beswick and Evanson 1957). Hultborn et al. (1996) demonstrated that this presynaptic inhibitory mechanism is not accompanied by GABAergic primary afferent depolarization and does not produce dorsal root potentials. They also concluded that this inhibition does not spread...
to the motoneuron pool and is strictly confined to those Ia fibers which have been previously activated.

PAD has also been examined in pathologic conditions. It is known that this depression is substantially reduced in spastic patients, patients with multiple sclerosis and spinal cord injuries (Grey et al. 2008; Nielsen et al. 1995). Evidence also exists to suggest that the impairment in PAD contributes to the pathophysiology of spasticity and also correlates with hyperreflexia (Aymard et al. 2000). Yet, the exact mechanism which causes a reduction of PAD in pathology is not well understood. In these pathological conditions, the decrease of depression is more pronounced as the interval between the two stimuli increases (faster recovery of the test H-reflex). On the other hand, in pathological conditions there is limited information regarding the effect of an unequal size conditioning H-reflex on the amplitude of the test H-reflex. In this study we deviated from the traditional PRD method by varying the amplitude of the conditioning H-reflex against a constant test H-reflex. This new method enabled us to examine the interaction between the stimulus interval and the conditioning stimulation intensity and provided a new aspect of the pathophysiology of post activation depression.

Methods

A total of 10 healthy (age = 26.4±3.4 yrs) and 10 stroke patients (age = 63.5±12.0 yrs) participated in this study. Healthy subjects self-reported of having no neurological disorder. In this group, all measurements were performed on the right lower extremity. Hemiplegic stroke subjects had suffered from one cerebrovascular accident ranging from 2 months to 5 years prior to the time of data collection. In this group all measurements were performed on the affected side. All subjects read and signed the consent form approved by the Institutional Review Board of Indiana University Bloomington and Indiana University Health La Porte Hospital.
A Therapeutic Unlimited® EMG unit was used for electromyographic (EMG) data recording. A pair of Ag-AgCl surface electrodes with an internal diameter of 0.5 cm and a fixed inter-disc distance of 2 cm was placed above the Achilles tendon and over the soleus muscle and another pair of electrodes was placed on the motor point of the tibialis anterior muscles and parallel to its fiber orientation. The ground electrode was placed on the lateral malleolus. The EMG signals were digitized at 4000 Hz using a National Instrument A/D board. Two constant current units (Digitimer DS7A, Hertfordshire, UK) were used for nerve stimulation. A two-channel Grass stimulation unit was used to control the precise timing and the sequence of triggering of the two constant current stimulators.

The soleus H-reflex was elicited by placing a pair of stimulating bar electrodes with a fixed inter-disc distance of 2 cm over the posterior tibial nerve (PTN). Maximum muscle response (M-max) and maximum H-reflex (H-max) was measured. The amplitude of the H-reflex was set to be between 15% to 30% of M-max. The small M-waves on each trial were monitored to ensure consistent stimulation conditions. After each nerve stimulation (paired reflex or single pulse) at least a 10 second delay was given to allow full recovery of the H-reflex (Crone and Nielsen 1989; Knikou 2008).

Paired reflex depression (PRD) was used to assess PAD (Robertson and Koceja 2003) in all ten healthy individuals and all hemiplegic patients. In this method, two stimuli were delivered to the PTN with intervals of 80, 150, 200, 250 and 300 ms between the conditioning and the test reflexes (fig 1A). At least five trials were elicited at each interval. When M-wave amplitude fluctuated more than 5% the trial was repeated. The ratio of the test H-reflex to the conditioning H-reflex was measured at each tested interval to determine the amount of depression. For the statistical analysis of this part of the study, a two way ANOVA (Group × Interval) was used.
(Keppel and Wickens 2004). Subsequently one sample t-tests were used to compare the test/conditioning H-reflex ratio to 1.0 to determine the presence of post-activation depression at the 5 selected intervals of 80, 100, 150, 200 and 300 ms (Cressie 1980).

To determine whether the ratio of the test/conditioning H-reflex in the conventional PRD method was affected by the amplitude of the reflexes, we performed the PRD method at different stimulation intensities. We used the same method illustrated in figure 1A but started from very low stimulation intensities and increased the intensity in a stepwise fashion until H-max was evoked. Any change in the inhibition ratio due to stimulation intensity would be a potential confounding factor for the second part of the study (explained in detail below). This preliminary part of the study was performed on 4 of the 10 healthy subjects and was tested at a fixed conditioning-test interval of 200 ms. For this experiment, the reflexes were normalized to M-max to assess whether the inhibition ratio was affected by the stimulation intensity, and allowed us to use the correct stimulus intensity for the second part of this study.

In the second part of the study, we deviated from the conventional PRD method by delivering each pulse from one of the two stimulation units and thus had the ability to change the intensity of the first stimulation while maintaining the second at a constant intensity (fig 1B). In this protocol, we first set each stimulation unit separately to elicit equal size H-reflexes. After eliciting several single H-reflexes from each unit and ensuring that both units would produce the same amplitude reflex, the two units were then sequenced to test the PRD at a fixed interval. After eliciting a few trials with the same intensities, the stimulation intensity for the conditioning reflex was altered and at least five trials were elicited at that intensity. Subsequently the amplitude was altered again and a few trials were elicited until enough conditioning H-reflexes with amplitudes ranging from nearly zero to 30% of M-max were elicited. In this alternative
protocol, therefore, the test H-reflex was influenced by a variable conditioning H-reflex. The reflexes were then normalized to M-max and were plotted against each other.

This protocol was conducted on 6 of the 10 subjects in the healthy group. The interval between the conditioning and the test reflex was 300 ms in this experiment and the amplitude of the control H-reflex was set to between 15-30% of the M-max. This protocol was also conducted in 9 hemiplegic patients at two different intervals of 80 ms (a short interval) and 300 ms (a long interval). In order to classify these two groups of data points (short interval vs. long interval) quantitatively, we used Fisher Linear Discriminant (FLD) analysis (Alpaydin 2004; Fisher 1936). FLD is one of the oldest, yet commonly used methods for discriminating data of different classes quantitatively. For two-dimensional data, the main idea is to find projections of samples from the known classes (two classes in our problem) onto a line that are optimally separated. The objective function ($J$-Value) to be maximised is defined to be the ratio of ‘between class scatter’ over ‘within class scatter’. The value of $J$ quantitatively describes how well the known classes are separated. A detailed mathematical description of the FLD method is given in Appendix A.

**Results**

PRD was tested in 10 healthy and 10 stroke patients at five different conditioning-test intervals (as illustrated in fig 1A). While both groups showed depression of the test reflex at short intervals, in the hemiplegic group the depression showed almost full recovery at the 300 ms conditioning-test interval (fig 2). Statistical analysis showed a significant interaction between Subject Group and Interval ($F_{3,54}=3.90$, $p=0.014$). There was a significant main effect of the Intervals ($F_{3,54}=28.12$, $p<0.001$) and also for the Subject Group ($F_{1,18}=30.53$, $p<0.001$). Post-hoc
analysis with the alpha level correction showed that the two groups were different at all conditioning-test intervals, revealing that hemiplegic patients had substantially less depression at each of the tested intervals. The existence of a statistical interaction between the two groups strongly suggests that the recovery of PAD is substantially different in these two groups. To infer at what interval the observed ratio was statistically equal to 1.0 (i.e., no substantial PAD), one sample t-tests were performed. The results of this test in the hemiplegic patients showed that the ratio was significantly less than 1.0 at 80 ms conditioning-test interval ($t=7.62$, $p<0.0001$) was still depressed at 150 ms ($t=2.66$, $p=0.026$), but was not significant at the longer-latency intervals. Therefore, at short latency intervals PAD existed in these patients but to a lesser extent than the healthy group.

PRD was tested in four healthy subjects with a fixed conditioning-test interval of 200 ms at various stimulation intensities to test the effect of the size of the conditioning H-reflex on PAD (with the protocol illustrated in fig 1A). By increasing the stimulation intensity for eliciting paired reflexes, larger reflexes were elicited and the ratio was calculated. It was observed that performing the PRD method with intensities which elicited conditioning H-reflexes between 15-30% of M-max the ratios were constant. Figure 3A shows the data from a representative subject. The slope of this curve is the test/conditioning H-reflex ratio and was perfectly linear at the range delimited in figure 3. The inset is the fitted line for the four subjects. The mean $R^2$ for these four subjects was $0.84\pm0.098$. Figure 3B shows the pooled results of the ratio. Therefore, since in the conventional PRD method, the ratio was constant when the conditioning H-reflex was between 15-30% of M-max, we selected this amplitude for the next experiment (explained below). Also, a
nonlinear relation in the ratio (different sensitivities of the H-reflex to depression) would be a confounding factor, which was ruled out here.

To further investigate the pathophysiology of PAD, we used the alternative protocol at which an unequal size conditioning H-reflex was elicited against the test H-reflex (e.g., changing the amplitude of the conditioning H-reflex). In the healthy group, since the test H-reflex was substantially depressed at all intervals, we used a long ISI for this alternative protocol. This protocol was tested on six of the healthy subjects. Increasing the amplitude of the conditioning H-reflex produced a decrease in the size of the test reflex (fig 4A). Plotting the amplitudes of the test H-reflex against the variable conditioning H-reflex revealed a nonlinear pattern of change in the size of the test H-reflex as a result of increasing the amplitude of the conditioning H-reflex. These results are shown in figure 4B for all healthy subjects. The nonlinearity of the change between the two variables was determined by fitting the data points with a linear and an exponential model and measuring the goodness of fit (least squares). Fitting the data points obtained from this alternative method with a linear model yielded an $R^2=0.53\pm0.09$ while a nonlinear exponent yielded $R^2=0.79\pm0.1$. A statistical comparison between the linear $R^2$ ($lR^2$) and exponential $R^2$ ($eR^2$) showed that $eR^2$ significantly explained more of the variability of the data and was a better fit ($F_{1,5}=37.41\ p=0.002$).

We conducted the same alternative protocol in 9 stroke subjects. The protocol was conducted with two different conditioning-test intervals (as illustrated in fig 1B): a short interval
at which maximum PAD had been observed and a longer interval at which no inhibition was observed. Figure 5 shows the data of one exemplar hemiplegic subject. As can be seen in this patient, the ratio was below 1.0 at short intervals (fig 5A). Testing this ratio with different intensities at a short interval (in this subject at 100 ms) with the conventional PRD method (fig 1A), yielded a linear relation between the two. With most of the data points being above the identity line, the existence of PRD was indicated (fig 5B). Testing with the alternative method at this short interval also produced an exponential relation similar to that of healthy individuals (fig 5.C). However, as the conditioning-test interval increased, the amount of inhibition of the test H-reflex decreased and eventually yielded a test reflex larger than the conditioning reflex (fig 5A, at intervals > 250 ms). Testing the ratio at different intensities at a longer interval (in this patient at 300 ms) resulted in a linear relation with the data points being on or below the identity line (fig 5D), suggesting an exaggerated test H-reflex. Using the alternative method at this longer interval yielded a completely different relation (fig 5E). Comparing figure 5C with fig 5E suggests that conditioning the test H-reflex with a variable conditioning H-reflex yields different results depending on the timing between the two stimuli; at short intervals, it causes depression while at a long ISI, it can even cause facilitation. The only difference between these two conditions is the interval between the two reflexes.

To quantitatively measure this difference between the two conditions, FLD was used (details explained in the Methods and Appendix A). Figure 6A shows the results for both conditions in one stroke patient and figure 6B shows the J-value of all 9 stroke subjects. A one sample t-test showed that the J-value (separation between the two conditions) was significantly
above zero ($t=2.66, p=0.029$). This analysis suggests that these two patterns (classes of data) are significantly different from each other.

Discussion

PAD was examined in healthy and hemiplegic subjects by using PRD at various conditioning-test intervals. We observed that in the hemiplegic patients, PAD was generally reduced and also had a much faster recovery; i.e. the depression completely disappeared at the conditioning-test interval 300 ms. In healthy individuals, a full recover takes up to 10 seconds (Crone and Nielsen 1989). The characteristics of PAD were further examined by using an alternative protocol in which the amplitude of the conditioning reflex was different from that of the test reflex. In healthy individuals, increasing the size of the conditioning reflex exponentially decreased that of the test reflex. In the patients group, however, the changes in the test reflex were dependent on the conditioning-test interval; we observed that performing the same protocol at short intervals produced a pattern similar to that of healthy subjects. However, performing exactly the same protocol at a longer interval produced a pathologic response in these patients.

As is shown in previous studies which have used the H-reflex method for conditioning protocols such as recurrent inhibition, reciprocal inhibition and cutaneous inhibition (Crone et al. 1990), our data also showed that using an H-reflex with an amplitude between 15% to 30% of M-max is a suitable size for this study since the ratio of test/conditioning H-reflex was fairly constant in this range. As was expected, in the healthy group the ratio of test/conditioning H-reflex was significantly below 1.0 up to 300 ms. However, it is known that the depression of the test H-reflex can last up to several seconds after the conditioning stimulation (Clair et al. 2011;
Crone and Nielsen 1989; Robertson and Koceja 2003; Robertson et al. 2012). With our new method of using a conditioning H-reflex of variable size for the PRD method a nonlinear pattern emerged in the healthy group; increasing the size of the conditioning H-reflex exponentially decreased that of the test H-reflex. One possible explanation for this behavior is that by increasing the stimulation intensity of the conditioning reflex, the synapses of larger size motoneurons are being activated and therefore in the subsequent stimulation are more likely to be depressed. Another possibility is that the exponential change in the amplitude of the test H-reflex is partly related to the type of muscle fibers and the size of the motor unit being affected. Also, the strength of stimulation may affect the number of motoneurons being at the subliminal fringe (Pierrot-Deseilligny and Mazevet 2000). However with a higher intensity, these motoneurons are being recruited. As such, there is a possibility that some of the Ia fibers which also make synapses with these motoneurons are likewise subject to depression and therefore, exponentially affect the size of the test reflex. A final possibility is that the activation of Ia fibers affects other Ia fibers which were not involved in the first stimulation but were being activated by the second stimulation. None of these possibilities are mutually exclusive. Although, it was suggested in the 1960s that PAD activates long loop reflexes (Taborikova and Sax 1969), later intracellular experiments suggested that this type of inhibition does not spread to other Ia fibers (Hultborn et al. 1996). Regardless of the physiological reason for this phenomenon, we established normative data to compare with the pathologic condition.

In the hemiplegic group, the linear relation of test/conditioning H-reflex at various intensities (conventional method- fig 1A) also held with the difference that at longer intervals, the ratio was close to or even above 1.0 (fig 5B and D). Reduced PAD has been reported previously in pathologic and spastic conditions (Grey et al. 2008; Nielsen et al. 1993; Nielsen et
This impairment is also suggested to be correlated with spasticity and motor impairment (Burke et al. 2013). However, it is not well understood how this depression is related to the exaggeration of the stretch reflex.

Initial experiments on presynaptic inhibition with primary afferent depolarization suggested that this mechanism is reduced in hemiplegic spastic conditions. Subsequently, it was believed that the increase in neurotransmitter release is due to the impaired presynaptic inhibition. However, later studies have shown that presynaptic inhibition is intact or minimally impaired in upper motoneuron lesions (Aymard et al. 2000; Burke and Ashby 1972; Faist et al. 1994; Katz 1999; Lamy et al. 2009). Nielsen et al. (1995) suggested that stretch reflex hyperexcitability is likely due to mechanisms other than presynaptic inhibition. These mechanisms may contribute to changes in the efficacy of transmission on the Ia-motoneuron synapse. Anatomical changes and neuronal reorganization such as establishment of new connections might also contribute to the exaggeration of reflexes (Dietz and Sinkjaer 2007). This study provided new insight into the pathophysiology of the exaggerated reflex in hemiplegic patients. Our results suggest that the interval between the conditioning and the test reflexes plays a critical role in the pathology. We observed that increasing the amplitude of the conditioning reflex caused an exaggerated test reflex mainly at long intervals while at short intervals the pattern was similar to that observed in healthy individuals. This finding and the discrepancy between the effect of a short and a long interval on the test H-reflex cannot be readily explained by an increase of neurotransmitter release. Under normal physiological conditions, as time elapses, more glutamate transmitters are being restored by high affinity sodium-dependent glutamate transporters (Danbolt 2001). More importantly, it has been shown in the cat that baclofen reduces transmitter release in the Ia-alpha motoneuron synapses (Jimenez et al. 1991),
but its administration in spastic patients with MS did not increase the reduced PAD (Ørsnes et al. 2000). To the best of our knowledge, no study has been conducted to determine whether the reuptake mechanism is impaired in hemiplegic stroke. Therefore, with our current understanding, it is likely that mechanisms other than the increase in neurotransmitter release also contribute in the time-dependent exaggeration of the test H-reflex. Considering the complex collateral arborization of Ia fibers with the dendrites of alpha motoneurons (Brown and Fyffe 1981; 1978; Lev-Tov et al. 1983), it is likely that the activation of Ia fibers invades other Ia synapses in normal physiologic conditions and this invasion of action potentials might be reduced in pathologic conditions. However, with our current data, this assumption remains at the hypothetical level.

Modern theories of the pathophysiology of spasticity and hyperreflexia suggest that part of the issue is the alteration in muscle fibers and properties secondary to the CNS damage (Dietz and Sinkjaer 2007). There is a possibility, therefore, that the observed exaggerated reflex response could be due to muscle potentiation at long intervals where the muscle properties have enough time to cause potentiation (Xenofondos et al. 2014). However, with the current experimental design we are unable to differentiate between spinal pathologies and muscular pathologies. More importantly, the current study investigated the reflex pathway passively. While we observed a clear time-dependent impairment in PRD, it cannot be inferred how and to what extent this impairment contributes to movement disorders associated with hemiplegia.

Conclusion

The results of this study showed a more complex aspect of the pathophysiology of post-activation depression in hemiplegia than originally observed. It was demonstrated that the time
interval between the conditioning and the test reflex plays a critical role in producing a normal or
a pathological response in stroke patients. Our alternative protocol which consisted of using a
variable conditioning H-reflex provides new insight into the pathophysiology of PAD in
hemiplegia; in the absence of any other changes in the conditioning protocol and only changing
the interval between the two stimuli alters a normal pattern to an abnormal pattern in hemiplegic
stroke patients. Therefore, it appears that the timing of Ia fiber activation has a critical role in the
pathology of hemiplegic spasticity. This finding could be due to an impairment in the spread of
PAD in the motoneuron pool. This alternative approach might prove beneficial in examining the
effectiveness of post-stroke intervention protocols which target spinal cord pathways.

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Legends

Figure 1. Experimental setup and data collection protocols. In the conventional method (panel A), two stimuli were delivered to the nerve at various intervals (ISIs). Also, with the same setup and protocol, the stimulation intensity was increased to obtain the ratio of conditioning H-reflex/test H-reflex at various percentages of M-max. In the alternative method (panel B), the two stimuli were delivered from two different constant current units and merged into a single pair of cables by a customized bridging cable. By changing the stimulation intensity of the first channel, different amplitudes of the conditioning H-reflex were elicited to a constant intensity test reflex.

Figure 2. Paired reflex depression at the five examined paired reflex intervals. Each point is the average of all subjects with the standard error of mean. Asterisks show intervals at which the ratio was significantly below 1.0 (existence of PAD).

Figure 3. Examining the relation between the conditioning H-reflex and the test H-reflex at different intensities. Using a single constant current unit, two equal size stimuli were delivered to the nerve. Therefore, the ratio was tested at different percentages of M-max. (A): The results of the responses elicited with the same intensity. In the case of no inhibition at any of the intensities, all the data points would fall on the identity line. Therefore, a shift to the left and up (above the identity line) represents depression of the test H reflex. By increasing the intensity, it was observed that when the first H-reflex is between 15-30% of M-max a linear relation exists in the amount of depression (delimited by the square). The inset shows the regression lines for the four subjects. All lines fall above the identity line. (B) depicts the pooled data. The ratios were
categorized based on the intensity of the conditioning H-reflex as a percentage of M-max. Keep
in the mind that the stimulation intensity for both reflexes was the same. It can be seen that the
ratio is not being affected by the size of the conditioning H-reflex when the reflex is between 15-
30% of M-max.

**Figure 4.** Relation between various amplitudes of the conditioning H-reflex against a constant
amplitude test H-reflex in the healthy group. (A), The waterfall depicts individual traces for a
healthy subject. By increasing the amplitude of the conditioning H-reflex, the amplitude of the
test H-reflex decreased. (B), pooled results for all subjects. Each line/color presents one
participant. The ordinate shows the amplitude of the conditioning reflex and the abscissa shows
the resultant test H-reflex. It can be seen that when the test H-reflex is being conditioned by a
smaller conditioning H-reflex, the amount of inhibition exerted on it changes in a non-linear
fashion.

**Figure 5.** Results of PRD in an exemplar hemiplegic patient. (A): PRD obtained at various
conditioning-test intervals. The selected short and long interval are shown by single and double
arrows, respectively. These intervals are used for the subsequent tests. (B): test/conditioning H-
reflex ratio at different stimulation intensities at a short interval. (C): The results of the
alternative protocol. The exponential pattern is clearly observed. (D): Same test as (B), but with
the long interval. (E): Same test as (C) but with the long interval. Here, the exponential pattern is
lost and the test H-reflex is equal or bigger.
Figure 6. Fisher linear analysis results. (A): The method is shown in one subject. The arrow points to the optimal line that separates the projected data as well as possible. (B), J-values (separation between the two conditions) of all stroke patients.
References


Figure 2

Paired Reflex Depression at different ISIs

- Healthy
- Patient

Test/ConditioningH-reflex ratio vs. ISI (ms)

* denotes significant difference.
Figure 5

PRD at different ISIs

A

B

C

D

E

Short ISI experiment

Long ISI experiment
Figure 6

A

![Graph A](image)

- Conditioning H-Reflex (% of M-Max)
- Test H-reflex (% of M-Max)
- Opt line
- Short ISI
- Long ISI

B

![Graph B](image)

- Fisher Linear Discriminant Analysis
- J-Values
- Patients
- Patients 1 to 9